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Neurofibromatosis type 1 affects 1/4000 individuals worldwide and predisposes to the growth of both benign and malignant tumors. Our research is focused on NF1 microdeletions that are associated with an early onset, and subsequent heavy burden, of cutaneous neurofibromas and predispose to MPNST. We found that these deletions arise by homologous recombination between 51 kb repeat elements (NR1REP) that flank the NF1 gene. We identified recombination hotspots where 69% of NF1 microdeletions occur and developed robust and sensitive assays to detect microdeletions in a patient blood sample. We analyzed the structure and sequence of four NF1REP paralogs in the genome and described sequence features that may mediate recombination at these sites. We developed new quantitative PCR assays that will detect nonrecurrent NF1 microdeletions that occur either in the germline or in somatic tissues including tumors. Our data make substantial contributions to understanding how NF1 microdeletions occur, create important assays and resources to determine whether some individuals are more susceptible, and which deleted sequences may cause the severe tumor phenotype of these patients.

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Introduction

Neurofibromatosis type 1 affects 1/4000 individuals worldwide and predisposes to the growth of both benign and malignant tumors. We propose that the early age at onset of cutaneous neurofibromas observed in patients with *NF1* microdeletions is caused by the co-deletion of *NF1* and a second gene *NPL* (neurofibroma-potentiating locus)(1-3). Our findings that the majority of *NF1* microdeletion breakpoints are clustered at large repetitive elements (NF1REPs) that flank the *NF1* locus and thereby delete virtually the same set of genes supports our proposal (4). In this application, we proposed to test the following hypotheses: [1] *NF1* microdeletion breakpoints occur at a small segment that defines a meiotic recombination hotspot(s) within the 15-100 kb NF1REP elements and that homologous recombination at the hotspot is facilitated by a nearby recombinogenic element. [2] Polymorphism in NF1REP number, orientation, and/or complexity predisposes certain individuals to *NF1* microdeletion and the consequent high neurofibroma burden. [3] *NF1* microdeletion increases the risk of developing a solid tumor malignancy. [4] NF1REP-mediated *NF1* microdeletion in somatic cells is an underlying mechanism of loss of heterozygosity at the *NF1* locus in malignant tumors of NF1 patients. This research will identify specific genetic loci and mechanisms that play a role in tumor development in NF1 patients, elucidate the mechanism of deletion, and develop assays for rapid and sensitive detection of NF1 microdeletions that may be applicable in the clinical diagnostic setting.

Body

Progress described for items/timeline described in the original "Statement of Work" in the grant proposal.

Year 1:

- **Development of a probe to detect NF1REP-mediated *NF1* deletion junction fragments**

We have mapped and sequenced the breakpoints of deletion patients and identified two recombination hotspots for recurrent NF1 microdeletion, along with mapping and sequencing three novel breakpoints. We have developed rapid and accurate polymerase chain reaction (PCR) assays that can detect the recurrent NF1 microdeletions in a blood sample from a patient. These results far exceed our original statement of work, when we envisioned development of a probe for use on Southern blots to detect *NF1* microdeletion junction fragment. Together, these data have confirmed and proven our first hypothesis that *NF1* microdeletion breakpoints occur at a small segment that defines a meiotic recombination hotspot(s) within the 15-100 kb NF1REP elements and that homologous recombination at the hotspot is facilitated by a nearby recombinogenic element. These results are published {Lopez-Correa, 2001 #939; Stephens, in press #1001; Dorschner, submitted #1270} (see Appendix).

We have shown that the majority of *NF1* microdeletions are 1.4 Mb in length and arose by a mechanism of recombination between misaligned directly-oriented repeat elements that flank the *NF1* gene [termed NF1REP-P1 (proximal) and -M (medial)] (4)(Figures 1, and 2). These repeats are paralogs, which are sequences of high identity that arise by duplication within a species. We introduced the term paralogous recombination to describe the process of homologous recombination between paralogs (5). Identification and characterization of the genes within the deleted region are detailed given in our manuscripts (4, 6, 7)(see Forbes et al and Jenne et al manuscripts in the Appendix).

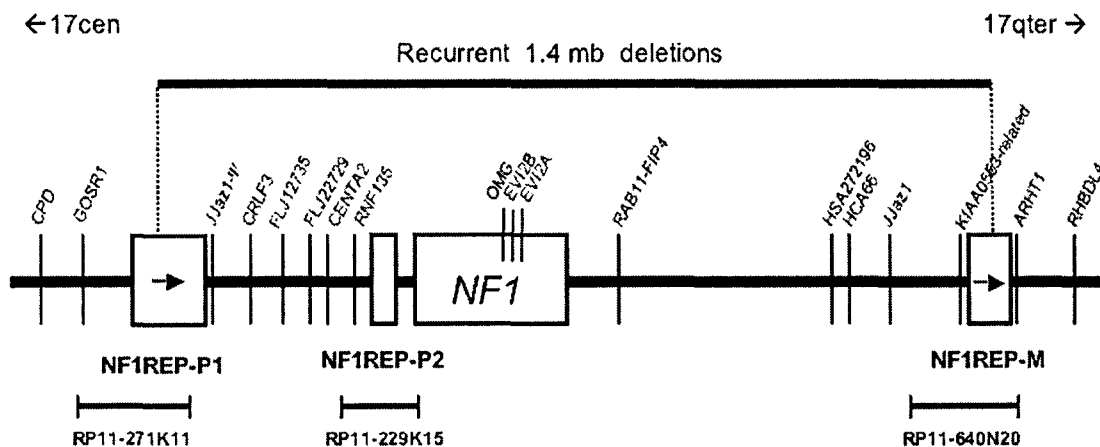


Figure 1. Schematic of the paralogous recombination sites within the NF1REP paralogs. Paralogous recombination between the NF1REP elements results in a recurrent 1.4 Mb deletion of the entire *NF1* locus. The location of the paralogous recombination sites, PRS1 and PRS2, are shown. The stippled portion of PRS2 designates the 2 kb recombination hotspot that we reported previously (8), while the hatchmarks designate the extended portion site (Dorschner et al., in preparation, see below). The basepair coordinates are relative to BAC 271K11, which contains NF1REP-P1.

We mapped *NF1* deletion breakpoints to the sequence level, we unexpectedly discovered that 46% of patients with entire *NF1* gene deletions (N=54) have breakpoints that map to a 2 kb recombination hotspot within the NF1REPs (8)(see Appendix) (Figure 2 below). A PCR assay to detect deletion junction fragments was developed by locating the upstream primer in NF1REP-P1 and the downstream primer in NF1REP-M taking advantage of paralogous sequence variants (PSV, which are NF1REP-specific nucleotide differences) between the two paralogs. A 3.4 kb amplicon is produced from deleted chromosomes with breakpoints at this hotspot; however, amplification of the 1.5 Mb segment from normal chromosomes cannot occur.

By mapping and sequencing *NF1* microdeletion breakpoints of additional patients, we have extended the location of this original *NF1* microdeletion hotspot and have identified a second hotspot, developed assays to detect deletions at both hotspots, and determined the frequency of *NF1* microdeletion that occurs at these hotspots. These data are described in detail in the attached manuscript Dorschner et al, which is being revised for resubmission {Dorschner, submitted #1270}(see attached). In summary, the first hotspot was extended to 4.1 kb in length and designated as paralogous recombination site 1 (PRS1). The second cluster was 6.3 kb in length and extended the cluster harboring the previously defined 2 kb recombination hotspot. This cluster, designated as paralogous recombination site 2 (PRS2), was about 15 kb telomeric to PRS1. We developed new, efficient PCR assays that detect *NF1* microdeletions at either PRS1 or PRS2. We applied these assays to

screening a larger cohort of NF1 deletion patients (N=78) and determined that 18% of deletions occurred at PRS1, while (51%) occurred at PRS2 (9)(see Appendix). As expected, neither the 7 kb PRS1 nor the 7 kb PRS2 deletion-junction assays generated products upon amplification of 150 normal chromosomes. These assays can be used in clinical laboratories for the diagnosis of NF1 patients that carry the recurrent 1.5 Mb *NF1* microdeletion and in research laboratories to identify patients for phenotype/genotype studies.

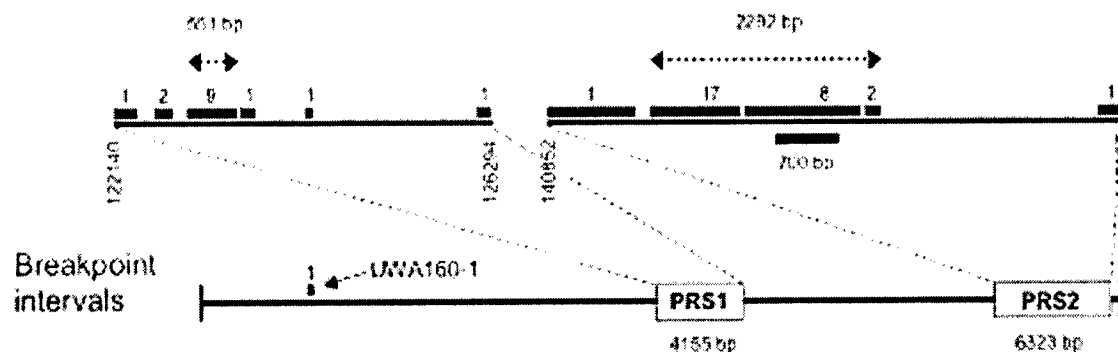


Figure 2. Clustered NF1 microdeletion breakpoints at two distinct paralogous recombination sites (PRS). Breakpoint intervals are shown along with the 2.3 kb hotspot, which harbors 93% of breakpoint intervals in PRS2 region. Finer localization of the 700 bp gene conversion tract and the promoter like sequences from panel A are shown. Nucleotide positions for both panels refer to the NF1REP-P1-51 in BAC RP11-271K11. Figures adapted from Forbes et al., (6) and reviewed by Stephens (10); see Appendix.

Breakpoints of mitotic NF1 microdeletions do not occur at PRS1 or PRS2, but at distinct sites.

In addition to these studies of germline NF1 microdeletions, we have determined breakpoints in patients who have somatic mosaicism for an NF1 microdeletion do not occur at the PRS1 or PRS2 hotspots. This implies that these microdeletions may occur by a different mechanism(s). This is important to determine since the somatic NF1 loss that occurs during tumorigenesis may occur by a similar mechanism. We have mapped two mitotic NF1 microdeletion breakpoint to the sequence level. Case UWA186-1 has a deletion that arose by paralogous recombination between JJAZ1-pseudogene and JJAZ1 functional gene (see Figure 1; Stephens et al. Manuscript in preparation). We developed a deletion junction fragment specific assay, and out of ~250 NF1 subjects screened and about 100 neurofibromas, we have not found another patient with a breakpoint in this region. However, recent reports document mitotic recombination between JJAZ pseudogene and function gene as a common mechanism of NF1 deletion mosaicism (11). The second mosaic case UWA208-1 maps between PRS1 and PRS2 (Forbes S., et al, manuscript in preparation). We also developed a deletion junction fragment specific assay for the UWA208-1 deletion breakpoint and after screening ~250 NF1 subjects screened and about 100 neurofibromas, we have not found other constitutional or mitotic breakpoints at this location.

NF1REPs are comprised of a complex modular arrangement of paralogs of different sequence families.

Mapping *NF1* breakpoints necessitated a detailed understanding of the structure and sequence of the NF1REP paralogs in order to design appropriate primers and avoid co-amplification of other paralogs. We have determined the structure of four NF1REP paralogs by STS mapping, sequencing, BLAST and BLAT analyses, and sequence alignments. Figure 3 below compares the structure of NF1REP-P1 and NF1REP-M, which act as paralogous recombination substrates for generating the 1.4 MB recurrent NF1 microdeletion. These data do not identify a sequence-specific motif responsible for the recombination hotspots, but rather indicate that multiple factors contribute to the location of the recombination events. These data are detailed in Forbes et al (6) and reviewed in Stephens K. (10)(see Appendix).

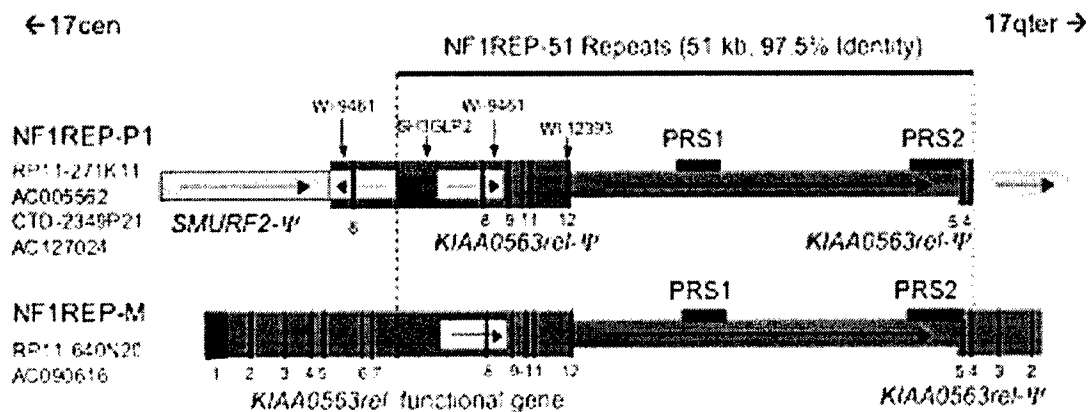


Figure 3. Comparison of the genomic structure of NF1REP-P1 and -M. Green color indicates sequences of the KIAA0563rel functional gene and related pseudogene fragments (Ψ), with numbered black vertical bars designating exons or related, exon-derived sequences. Light green boxes within KIAA0563rel copies indicate the orientation of a 5.8 kb inverted repeat with two copies on NF1REP-P1, and a third copy in NF1REP-M. Landmark STSs in the KIAA0563rel gene and pseudogenes cited previously (4) are indicated above NF1REP-P1. Yellow boxes depict SMURF2-derived pseudogene fragments, and red boxes denote the PRS. Figure from Forbes et al. (12).

Constructing a fine, sequence-based map of each NF1REP as described in Forbes et al., was necessary to facilitate experiments to test our second hypothesis that polymorphism in NF1REP number, orientation, and/or complexity predisposes certain individuals to NF1 microdeletion and the consequent high neurofibroma burden. We have examined the NF1REP-P1 and NF1REP-M structure and sequence of the parental chromosomes that underwent recombination to create the de novo *NF1* microdeletion in their affected child. We have constructed human-rodent somatic cell hybrids to separate the two chromosomes 17 of each of the parents of 4 cases. Analysis of such hybrid lines has identified the two chromosomes that were substrates for the recombination. Analysis of the NF1REP-P1 and NF1REP-M of these chromosomes has not revealed any polymorphism in NF1REP structure or orientation (Stephens K, data unpublished). Originally, we simply amplified NF1REP regions from the genomic DNA of each parent, cloned the amplimers and sequenced the region. However, we found unusual and inconsistent sequences that could not be easily explained. To determine if they were from polymorphism or unusual NF1REP structure, we embarked on the somewhat arduous task of constructing the hybrid lines. Direct sequencing of NF1REP from these lines will avoid cloning artifacts determined the precise structure of the parental NF1REP recombination substrates. Therefore, although our sample size is small, there is no evidence for NF1REP structural polymorphism that may predispose to microdeletion.

Gene and transcript map of the *NF1* microdeletion region.

In important resource for understanding the genetic basis of the more severe phenotype observed in *NF1* microdeletion patients is a complete physical map of the *NF1* deletion region at chromosome 17q112. In collaboration with Dr. Dieter E. Jenne (Max-Planck-Institute of Neurobiology), we have combined our map and sequence data and constructed a physical and transcript map of the >2 Mb *NF1* microdeletion region. These

data are detailed in the attached manuscript (7) and the functions of the genes are reviewed in Stephens K. (10)(see Appendix for manuscripts).

Years 2 and 3:

- **Ascertainment and sample collection of NF1 patients with tumors**
- **Employ the PRS1 and PRS2 *NF1* gene dosage assays to determine if the frequency of NF1 microdeletions is greater in patients that develop malignancies**

On May 12, 2003, the USAMRMC Human Subjects committee finally approved our protocol. The difficulty of getting this relatively simple protocol approved by this committee has been a major, and unexpected, disappointment of this study. To try to complete this portion of the study, we obtained a one year no-cost extension for this grant award.

We have identified 430 adults with malignant peripheral nerve sheath tumors (MPNST) that were referred to the University of Washington Medical Center (UWMC). Careful examination of the medical records revealed that 41 had neurofibromatosis 1. We have sent letters to their UWMC care providers and/or their primary referring providers to determine if the patients are alive and to forward a letter to them to determine if they are interested in participating in our study. In addition, we have ascertained 17 children affected with NF1 who had solid tumor malignancies, primarily optic glioma and MPNST. We are currently preparing materials to be sent to their care providers to determine interest in our study.

In addition, we are screening the tumor database at UWMC to identify NF1 patients who had solid malignancies other than MPNST. If sufficient patients are identified, we will also screen them for *NF1* microdeletions.

We simply did not have enough time to collect sufficient numbers of patients with malignancies to determine if NF1 microdeletion patients are predisposed to certain malignancies. These studies will continue, as they are a subset of the genotype/phenotype analysis we are undertaking in our current project DAMD17-03-1-0203. Our number of patients in this study will be larger due to a collaboration with Dr. Victor Mautner in Germany. Therefore, our work on this project will contribute to future studies.

Year 3:

Genotype grandparents to determine meiotic mechanism of microdeletion

For the following reasons, we have decided not focus on this one small aspect of our study. We will proceed to collect grandparental samples, but this aim may not be accomplished during the grant period. The reasons are:

1. We have little time remaining to obtain grandparental samples because of the extended length of time it took to obtain approval from the USAMRMC Human Subjects committee,
2. With the limited time we have left to accomplish the work, we have decided to focus our efforts the phase of the study regarding risk of malignancy
3. This aim is of less importance because another research group has investigated and published work related to this question (13).

Employ *NF1* microdeletion assays to detect NF1REP-mediated deletion junction fragment in tumor tissue of *NF1* patients

Hypothesis number 4 that NF1REP-mediated *NF1* microdeletion in somatic cells is an underlying mechanism of loss of heterozygosity at the *NF1* locus in malignant tumors of NF1 patients awaits our obtaining such tissues for analysis. These studies will continue, as they are a subset of a much larger genotype/phenotype analyses we are undertaking in our current project DAMD17-03-1-0203. Our number of patients in this study will be larger due to a collaboration with Dr. Victor Mautner in Germany. Therefore, our work on this project will contribute to future studies.

We have, however, disproven this hypothesis for benign neurofibromas. Using the PRS1 and PRS2 deletion junction-specific amplification assays, we have screened DNA from 171 cutaneous and plexiform neurofibromas and have not detected *NF1* microdeletions at these recombination hotspots (9). If somatic paralogous recombination is a mechanism of loss of heterozygosity at *NF1* during neurofibromagenesis, it occurs at novel sites.

Development of new quantitative PCR assays to detect novel *NF1* microdeletions that occur in germline or somatic tissues.

Since approval of our Human Subjects protocol was delayed much longer than we anticipated, we made valuable use of our time by developing new, highly sensitive, quantitative PCR assays to detect *NF1* microdeletions of any length in either germline or somatic tissues.

These assays required the capability and sensitivity to determine if a locus or gene has no deletion (2 copies), one gene deletion (1 copy), two gene deletion (0 copies), or amplification (likely >10 copies). Two gene deletion and gene amplification are simple to detect using various methods of quantitative PCR. The difficulty is in reliably differentiating one versus two copies of a gene. Therefore, we have been focusing on developing the best assay to detect a one gene deletion. To determine which quantitative PCR methods were sensitive, we choose to develop an assay at intron 31 of the *NF1* gene. To validate the assays we used DNA from normal individuals (2 *NF1* genes) and DNA from *NF1* patients with deletions involving the *NF1* gene (1 *NF1* gene). Initially, we tried using SYBR green (binds double stranded DNA) fluorescence as a method of detection during real-time PCR in the LightCycler instrument (Roche). There was considerable overlap between the crossing point values (Ct) for samples with one and two *NF1* genes indicating that the assay was not as sensitive or specific as required (data not show). Secondly, we sought to increase specificity and sensitivity by using a *NF1*- specific fluorescently-labeled primer. We constructed a LUX primer (Invitrogen), which is a hybrid primer comprised of *NF1* specific sequences and anonymous sequences that are capable of fold-back annealing. LUX primers are touted as having high specificity because they only fluoresce at high temperatures when the fold backs are melted. We had multiple problems with LUX primers and decided to abandon that approach (data not shown). The third method we developed was precise, sensitive, and specific and involves SYBR green for detection, competitive quantitative PCR, and melting curve analysis as detailed below.

We chose to employ SYBR green for detection in combination with competitive PCR, which is the most suitable method of quantification when highly accurate determinations are required. We adapted and modified a method published by Ruiz-Ponte et al. (14). In this method, a known copy number of a competitor is introduced directly in the PCR mixture along with the target DNA of the patient/tumor. The competitor, which is almost identical to the target DNA but distinguishable by product length, is amplified with the same set of primers so that efficiency of amplification for the two amplicons is the same. Calibration curves of different competitor concentrations determine the optimal concentration that equals that of the target DNA. Figure 4 below shows the melting curves of a normal control DNA samples (2 copies of *NF1*), where competitor and intron 31 are co-amplified with equal efficiency and the area under the curves are equal (roughly equivalent to peak height in this example). We constructed the competitor such that it would be amplified with the intron 31 primers, but have a different melting curve by replacing an internal TTT sequence with a CCC sequence. As expected, negative samples lacking human DNA did not amplify.

Figure 5 below shows the melting curve of a patient with an *NF1* deletion (1 copy of *NF1*) versus that of the normal control individual. Note that the amplitude of the melting curve of the *NF1* amplicon for the deletion patient is less than that of the competitor amplicon because there are fewer targets in the deleted patient's DNA. For precision, we use the peak areas of each melting curves for quantitation rather than peak height. A

ratio of peak area of normal control (2 copies) over the peak area of the patient target DNA is calculated, see below. (Wang B et al., unpublished data).

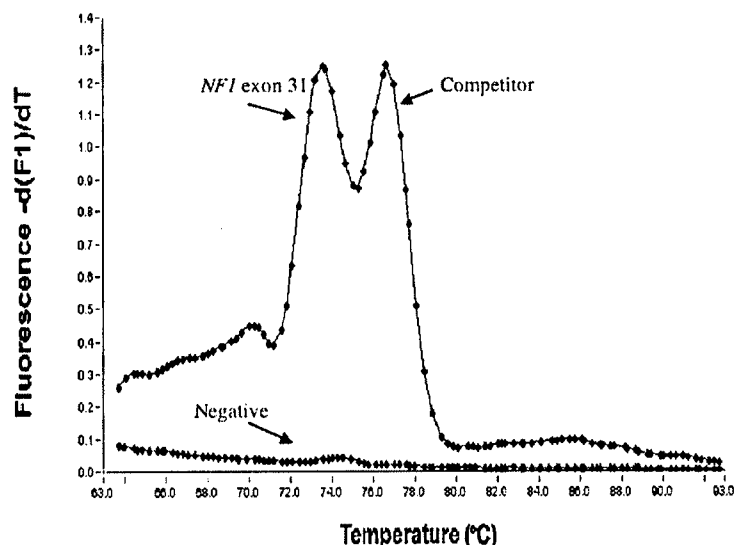


Figure 4. Melting curve analysis after competitive, quantitative PCR of NF1 intron 31 in genomic DNA of a normal control individual. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplification. (Wang B et al., unpublished data).

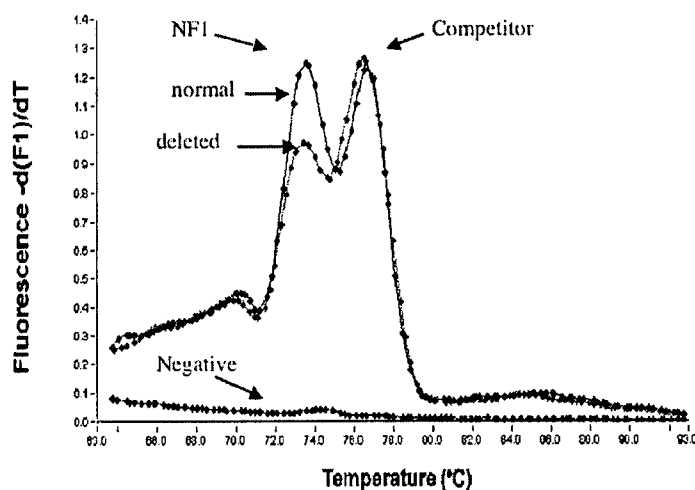


Figure 5. Melting curve analysis after competitive, quantitative PCR at NF1 intron 31 can differentiate one gene copy versus two gene copies. The results of two reactions are shown, closed circles represent target DNA from a normal control individual and closed squares represent target DNA from an NF1 patient with a deletion of one gene. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplification. (Wang B et al., unpublished data).

Once the concentration of competitor is determined for a certain concentration of the normal control DNA, it is essential that all subsequent reactions with unknown patient DNA samples contain exactly the same

concentration of target DNA. Prior to the competitive quantitative PCR assay, we determine the exact concentration of each patient sample using real-time quantitative PCR at a different locus. We amplify the TPA (tissue plasminogen activator) gene on chromosome 12 in each patient and compare that to a standard curve using the normal control DNA. From this reaction, we can calculate exactly what volume of patient DNA must be added to the competitive quantitative PCR assay. An example of the TPA real-time PCR and standard curve is shown in Figure 6. (Wang B et al., unpublished data).

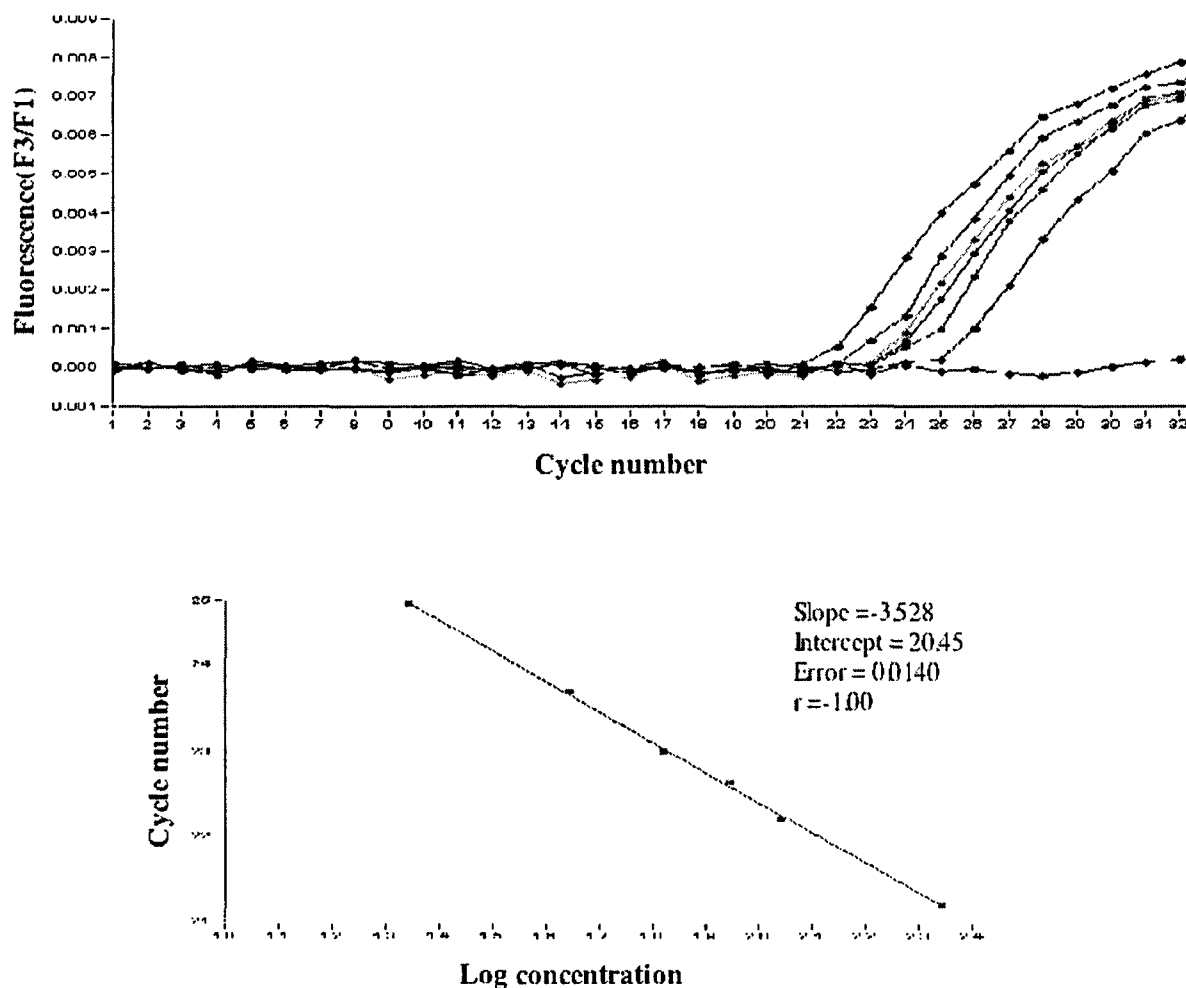


Figure 6. LightCycler real-time PCR of TPA locus showing standard curve. The upper panel shows the results of real-time PCR of the TPA locus of a dilution series of a normal control individual. The reaction consists of unlabeled primers and uses an internal labeled (fluorescence resonance energy transfer (FRET) probe for detection of product. The crossing point (Ct) is defined as the fractional cycle at which fluorescence begins to increase exponentially and is calculated by the LightCycler. Ct becomes larger as the number of TPA targets decreases. The lower panel shows the standard curve calculated from the above data. Note the low error and high correlation coefficient. (Wang B et al., unpublished data).

The development of additional competitive quantitative PCR assays. We developed additional assays spanning the *NF1* gene region. The assay for each locus were screened against an appropriate panel of monochromosomal human-rodent hybrid cell lines to ensure we are not amplifying homologous loci from other chromosomes. Location of the loci are shown in Figure 7.

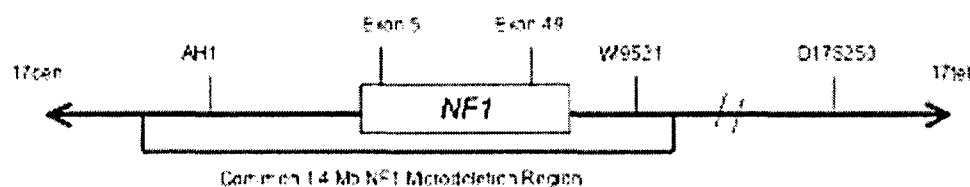


Figure 7. Location of loci at the 17q11.2 *NF1* region for which competitive, quantitative PCR assays were developed. Two loci are intragenic to *NF1* and two are flanking; all four are within the common 1.4 Mb *NF1* microdeletion region previously defined by us (8, 15). D17S250 is at 17q22 region and is never involved in *NF1* microdeletions; it is used as a disomic control locus.

The intra-assay and inter-assay variation of these assays is minimal as shown in Table 1 (Wang B et al., unpublished data). There is no overlap of values for disomic samples vs. monosomic samples. As expected, the disomic control locus D17S250 gave values of approaching 1.0 for both subjects.

Table 1. Variation of competitive quantitative PCR assays¹

Intra-assay variation (mean \pm 1 S.D.)					
Locus	AH1	<i>NF1</i> exon 5	<i>NF1</i> exon 49	WI-9521	D17S250
Normal subject	0.97 \pm 0.18	1.21 \pm 0.13	1.03 \pm 0.1	1.10 \pm 0.15	1.11 \pm 0.16
<i>NF1</i> microdeletion subject	0.33 \pm 0.05	0.52 \pm 0.13	0.51 \pm 0.11	0.44 \pm 0.13	1.15 \pm 0.18
Inter-assay variation (mean \pm 1 S.D.)					
Normal subject	0.95 \pm 0.13	1.23 \pm 0.26	0.97 \pm 0.16	0.98 \pm 0.17	1.07 \pm 0.15
<i>NF1</i> microdeletion subject	0.32 \pm 0.13	0.53 \pm 0.14	0.48 \pm 0.06	0.36 \pm 0.10	1.12 \pm 0.14

¹Intra-assay variation was determined by assay of 20 replicate samples in one experiment. Inter-assay variation was determined by assay of the same normal subject and deletion subject in 20 independent experiments performed on different days.

We have just completed screening several hundred *NF1* subjects for deletions involving the *NF1* locus using these assays (Wang B et al., unpublished data). Some of the deletions are still being confirmed by fluorescence

in situ hybridization (FISH), but our preliminary data are summarized below in Table 2 (Wang B et al., unpublished data).

Table 2. Identification of new microdeletion cases by quantitative, competitive PCR assays.

Result of deletion screen	Number of probands
No deletion at any of 4 loci	172
Deletions at PRS1 or PRS2	10
Deletion of AH1 and NF1 exon5, but not NF1 exon 49 or WI-9521	4
Deletions involving AH1, NF1 exons 5 & 49, and WI-9521 with unknown breakpoints	6
Affected family members have different NF1 mutations	3 families
Total	195

Two results are unexpected. First, we found 4 cases with novel deletions that involve a centromeric portion of the NF1 gene and an unknown number of genes centromeric to NF1. Such deletions have not been identified before. Microsatellite marker analysis has somewhat narrowed the deletions in these cases. To analyze precisely, we have just finished constructing somatic cell hybrid lines that are being analyzed. We hope that these cases may help to narrow the locus that predisposes NF1 microdeletion carriers to early onset and great numbers of cutaneous neurofibromas and to MPNST. Secondly, we found deletions in two individuals that were members of two unrelated families with multiple affected individuals. When the other affected relatives were tested, none were found to carry deletions. We are currently sequencing the other family members to identify their NF1 gene mutation, which is presumably intragenic. Because these were among a group of patients unselected for their phenotype, the frequency of families with multiple NF1 mutations segregating may be as high as 2%. This study will be completed as part of our current larger deletion genotype/phenotype study DAMD17-03-1-0203.

Identification of a new function of neurofibromin: caspase target.

During this project, we initiated a new collaboration with Drs. Melanie Kuechle, a caspase expert in the Division of Dermatology, University of Washington, and Dr. Michi Shinohara, a resident working with Dr. Kuechle. As detailed in the abstract Shinohara et al in the Appendix, we have evidence of caspase-mediated proteolysis of neurofibromin. This is an exciting new function that may play an important role in signaling and tumorigenesis. Dr. Kuechle is submitting a grant application to the DOD based on our findings.

Key Research Accomplishments

- We identified two recombination hotspots where 69% of germline *NF1* microdeletions occur. This is important because 1) these hotspots can be analyzed in detail to investigate why recombination is favored at these sites and 2) the majority of microdeletion patients will have virtually the same genotype and will therefore constitute an important patient cohort for genotype/phenotype analyses.
- We developed 5 different simple and reliable PCR assays that detect the presence of the recurrent 1.4 Mb germline *NF1* microdeletion in a patient blood sample.
- We showed that loss of heterozygosity in benign neurofibromas does not occur at the PRS1 and PRS2 *NF1* microdeletion hotspots of germline *NF1* microdeletions
- We showed that somatic mosaic microdeletions occur at different sites than germline microdeletions. This implies that they occur by a novel mechanism, which may also apply during tumorigenesis.

- We performed detailed structural and sequence analysis of the *NF1* paralogs, NF1REP-P1, P2, and M, which identified factors that could contribute to the recombination at hotspots PRS1 and PRS2.
- We developed a specific and sensitive competitive, quantitative PCR methodology that can differentiate one copy from two copies in a genome. We employed real-time PCR of the TPA locus as a means of precise DNA quantification. We developed and validated the competitive, quantitative PCR methodology by assay of two intragenic sites with the NF1 gene and in flanking loci of both normal control individuals and NF1 deletion patients.
- We screened about 200 NF1 probands for partial or full deletion of the NF1 gene and found novel partial deletions of the centromeric NF1 gene region, families with affected individuals who carry different NF1 gene mutations, and identified additional NF1 deletion cases for our ongoing project of deletion genotype/phenotype analysis.
- In collaboration with Drs. Kuechle and Shinohara, we have evidence of caspase-mediated proteolysis of neurofibromin (see abstract, Appendix).

Reportable Outcomes

Manuscripts, Peer-reviewed, primary research:

- Cooper LJN, Shannon KM, Loken MR, Weaver M, Stephens K, Sievers EL. Evidence that juvenile myelomonocytic leukemia can arise from a pluripotential stem cell. *Blood* 96:2310-2313, 2000. (see Appendix)
- López-Correa C, Dorschner MO, Brems H, Lázaro C, Clementi M, Upadhayaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns J-P, Marynen P, Stephens K, Legius E. Recombination hotspot in NF1 microdeletion patients. *Hum Mol Genet* 10:1387-1392, 2001. (see Appendix)
- Messiaen L, Riccardi V, Peltonen J, Maertens O, Callens T, Karvonen SL, Leisti EL, Koivunen J, Vandenbroucke I, Stephens K, Poyhonen M. Independent NF1 mutations in two large families with spinal neurofibromatosis. *J Med Genet*. 40:122-6, 2003. (see Appendix)
- Jenne DE, Tinschert S, Dorschner MO, Hameister H, Stephens K, Kehrer-Sawatzki H. Complete physical map and gene content of the human NF1 tumor suppressor region in human and mouse. *Genes Chromosomes Cancer* 37:111-20, 2003. (see Appendix)
- Forbes SH, Dorschner MO, Le R, Stephens K. Genomic context of paralogous recombination hotspots mediating the recurrent NF1 region microdeletion. *Genes Chromosomes Cancer* 41:12-25, 2004. (see Appendix)

Manuscripts submitted and/or in preparation

- Fishbein L, Sanek N, Stephens K, Wallace MR. An association between the ERBB2 codon 655 polymorphism and NF1. Submitted.
- Dorschner, M.O., Brems, H., Le, R, De Raedt T, Wallace MR, Curry CJ, Aylsworth AS, Haan EA, Zackai EH, Lazaro C, Messiaen L, Legius E, Stephens, K. Tightly clustered breakpoints permit detection of the recurrent 1.4 Mb NF1 microdeletion by deletion-specific amplification. In revision.
- Stephens K, Dorshner MO, Le R, Wallace MR, Forbes SH. Paralogous recombination breakpoint mapping in a case of somatic mosaic NF1 microdeletion. In preparation.

Invited Reviews:

- Stephens K. Genetics of neurofibromatosis 1-associated peripheral nerve sheath tumors. *Cancer Investigation* 21:897-914, 2003. (see Appendix)

Book Chapters:

- Stephens K, Tait JF, Jacobs DS, Garg U. Molecular Genetic Testing. In *Jacobs and DeMott Laboratory Test Handbook*, D.S. Jacobs, WR DeMott, DK Oxley, eds. Cleveland: Lexi-Comp Inc., 5th edition, pp 701-718, 2001.
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- Stephens K. Neurofibromatosis. In *Molecular Pathology in Clinical Practice*, Eds. D.G.B. Leonard, A. Bagg, A. Caliendo, K. Kaul, K. Snow-Bailey, V. Van Deerlin, Springer-Verlag, in press. (see [Appendix](#))
- Stephens K. Neurofibromatosis. In *Genomic Disorders: The Molecular Basis of Genomic Disease*. Eds. J.R. Lupski, PT Stankiewicz, New Jersey: Humana Press, in press. (see [Appendix](#)).

Abstracts

1. Stephens K. Molecular diagnosis of inherited skin disorders". Clinical Dermatology 2000, Vancouver, B.C., Canada
2. Stephens K, Dorschner MO, Friedman CL, Trask BJ, Sybert VP. 2000. Recombination hotspots for NF1 microdeletions. Am Soc Hum Genet, 2000, Philadelphia.
3. Stephens K, Dorschner MO, Leppig KA, Sybert VP. Evidence for a tumor-modifying gene in neurofibromatosis type 1. Ninth European Neurofibromatosis meeting, April 6-8, 2001, Venice, Italy.
4. Messiaen L, Riccardi V, Peltonen J, Callens T, Vandenbroucke I, Karvonen SL Stephens K, Poyhonen M. Mutation analysis in two large familial spinal neurofibromatosis families shows that the type of NF1 mutation can not explain the clinical homogeneity in severity of the disease. National Neurofibromatosis Foundation International Consortium for the Molecular Biology of NF1 and NF2, Aspen, CO, June, 2002.
5. Stephens, K. About 70% of NF1 microdeletions are recurrent and occur at discrete recombination sites within the flanking NF1REP paralogs, which are complex modular assemblies of low-copy repeats of different sequence families. National Neurofibromatosis Foundation International Consortium on Gene Cloning and Gene Function of NF1 and NF2. Aspen, CO, June, 2003.
6. Wang B, Bong S, Le R, Stephens K. Identification and Mapping of NF1 contiguous gene deletions using real-time competitive PCR. National Neurofibromatosis Foundation International Consortium for the Molecular Biology of NF1 and NF2, Aspen, CO, June, 2004. (see [Appendix](#))
7. Shinohara MM, Kuechle MK, Graves J, Stephens K. Caspase-mediated proteolysis of neurofibromin. Society for Investigative Dermatology, 2005. (see [Appendix](#))

Seminars, Invited Speaker:

- Stephens K. Continuing Medical Education Series: The Twentieth Century and the Impact of Technology on Clinical Genetics. "Mechanisms of Contiguous Gene Syndromes". Seattle, April 21, 2001.
- Stephens K. Ninth European Neurofibromatosis meeting, "Evidence for a tumor-modifying gene in neurofibromatosis type 1". April 6-8, 2001, Venice, Italy.
- Stephens K. National Neurofibromatosis Foundation International Consortium on Gene Cloning and Gene Function of NF1 and NF2. "Evidence for a tumor-modifying gene in NF1". Aspen, CO, June, 2001.
- Stephens K. University of Utah, Department of Pediatrics. "Paralogous recombination: mechanism of chromosomal deletion and duplication syndromes", June 18, 2001.
- Stephens K. University of Washington, Dept of Laboratory Medicine Grand Rounds, "Segmental deletions, duplications, and inversions: How do chromosomes do it?", Dec 5, 2001.
- Stephens K. Department of Medicine, Division of Medical Genetics, University of Washington, "Mechanisms and phenotypic consequences of recurrent, uniform NF1 contiguous gene deletions", February 22, 2002.

Stephens K. American College of Medical Genetics, Symposium on Chromosome Duplication Disorders, "Segmental duplication: How do chromosomes do it?", New Orleans, March 17, 2002.

Stephens K. Mayo Clinic, Division of Laboratory Genetics, Dept of Laboratory Medicine and Pathology, "Neurofibromatosis: Phenotype and Mechanism of NF1 Microdeletion", June 19, 2002.

Stephens K. National Neurofibromatosis Foundation International Consortium on Gene Cloning and Gene Function of NF1 and NF2. "Megabase Deletions", Aspen, CO, June, 2003.

Stephens K. Neurocutaneous Syndromes in the Developmental Age, "Molecular Genetics of NF1 and NF12", Fondazione Mariani, Lucca, Italy, March 3-5, 2004.

Invited speaker to lay organizations, Community Service

Stephens, K. NF Roundtable Discussion and Symposium, Washington Chapter of the National Neurofibromatosis Foundation, Seattle, WA, May 10, 2003, Panelist

Stephens, K. NF1 Research. Illinois Neurofibromatosis, Inc., Chicago, IL, October 19, 2003.

Development of research assays to detect NF1 microdeletion

Development of competitive, quantitative PCR assays for differentiating one versus two copies of NF1 gene intron 31.

Training

Michael O. Dorschner, Ph.D., Postdoctoral Fellow

Stephen H. Forbes, Ph.D., Postdoctoral Fellow

Bingbing Wang, Ph.D., M.D., Postdoctoral Fellow

Funding Awarded based on work supported by this award

4/7/03-4/6/07 US Army Medical Research & Materiel Command DAMD 17-03-1-0203
Clinical and Molecular Consequences of NF1 Microdeletion
Principal Investigator: Karen Stephens, PhD

Funding Application Pending based on work supported by this award

US Army Medical Research & Materiel Command, New Investigator Award
Title: caspase-mediated proteolysis of neurofibromin
Principal Investigator: Melanie Kuechle, M.D., University of Washington
Co-Investigator: Karen Stephens, PhD

Conclusions

Neurofibromatosis type 1 affects 1/4000 individuals worldwide and predisposes to the growth of both benign and malignant tumors. Genetic factors, in addition to defects in the NF1 gene itself, clearly play a role in tumor development. Our research is focused on identifying specific DNA sequences and genetic mechanisms important in the development of cutaneous neurofibromas, which occur in virtually all NF1 patients, and in the development of solid malignancies. We have analyzed NF1 microdeletions that are associated with an early onset, and subsequent heavy burden, of cutaneous neurofibromas. We determined the mechanism by which these deletions arise and identified recombination hotspots where 69% of NF1 microdeletions occur. We developed robust and sensitive assays to detect microdeletions in a patient blood sample; these assays can be directly applied in clinical diagnostic laboratories. We identified four NF1REP paralogs, analyzed their structure and sequence, and identified unique features that may mediate recombination at these sites. In addition, we

found that the NF1 microdeletions in some patients occurred early during embryonic development resulting in somatic mosaicism. We developed quantitative PCR assays to detect novel *NF1* microdeletions that occur either in the germline or in somatic tissues. Although only about 5-10% of NF1 patients carry microdeletions of the NF1 and adjacent genes, they are important to understand as these patients are at increased risk for neurofibromas and MPNST. An understanding of how *NF1* microdeletions occur, whether some individuals are more susceptible, and why they potentiate the development of neurofibromas is important for patient care, genetic counseling, and the design of effective pharmacological intervention strategies.

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6. Forbes SH, Dorschner, MO, Le, R and Stephens, K (2004) Genomic context of paralogous recombination hotspots mediating recurrent NF1 region microdeletion. *Genes Chromosomes Cancer*, 41, 12-25.
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Appendix

Pdf files of the following manuscripts/documents are attached and can be opened by double clicking on the icon.

Manuscripts, Peer-reviewed, primary research:

Cooper LNJ, Shannon KM, Loken MR, Weaver M, Stephens K, Sievers EL. Evidence that juvenile myelomonocytic leukemia can arise from a pluripotential stem cell. *Blood* 96:2310-2313, 2000.

López-Correa C, Dorschner MO, Brems H, Lázaro C, Clementi M, Upadhayaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns J-P, Marynen P, Stephens K, Legius E. Recombination hotspot in NF1 microdeletion patients. *Hum Mol Genet* 10:1387-1392, 2001.

Messiaen L, Riccardi V, Peltonen J, Maertens O, Callens T, Karvonen SL, Leisti EL, Koivunen J, Vandenbroucke I, Stephens K, Poyhonen M. Independent NF1 mutations in two large families with spinal neurofibromatosis. *J Med Genet.* 40:122-6, 2003.

Jenne DE, Tinschert S, Dorschner MO, Hameister H, Stephens K, Kehrer-Sawatzki H. Complete physical map and gene content of the human NF1 tumor suppressor region in human and mouse. *Genes Chromosomes Cancer* 37:111-20, 2003.

Forbes SH, Dorschner MO, Le R, Stephens K. Genomic context of paralogous recombination hotspots mediating the recurrent NF1 region microdeletion. *Genes Chromosomes Cancer* 41:12-25, 2004.

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Stephens K. Neurofibromatosis. In *Genomic Disorders: The Molecular Basis of Genomic Disease*. Eds. J.R. Lupski, PT Stankiewicz, New Jersey: Humana Press, in press.

Abstracts

Wang B, Bong S, Le R, Stephens K. Identification and Mapping of NF1 contiguous gene deletions using real-time competitive PCR. National Neurofibromatosis Foundation International Consortium for the Molecular Biology of NF1 and NF2, Aspen, CO, June, 2004.

Shinohara MM, Kuechle MK, Graves J, Stephens K. Caspase-mediated proteolysis of neurofibromin. Society for Investigative Dermatology, 2005.

Evidence that juvenile myelomonocytic leukemia can arise from a pluripotential stem cell

Laurence J. N. Cooper, Kevin M. Shannon, Michael R. Loken, Molly Weaver, Karen Stephens, and Eric L. Sievers

Children with neurofibromatosis type 1 (NF1) carry germline mutations in one allele of the *NF1* gene and are predisposed to myeloid malignancies, particularly juvenile myelomonocytic leukemia (JMML). Disruption of the remaining *NF1* allele can be found in malignant cells. Flow cytometric cell sorting techniques to isolate the malignant cell populations

and molecular genetic methods to assay for somatic loss of the normal *NF1* allele were used to study an unusual child with NF1 and JMML who subsequently had T-cell lymphoma. The data show that malignant JMML and lymphoma cells share a common loss of genetic material involving the normal *NF1* gene and approximately 50 Mb of flanking sequence, sug-

gesting that the abnormal T-lymphoid and myeloid populations were derived from a common precursor cell. These data support the hypothesis that JMML can arise in a pluripotent hematopoietic cell. (Blood. 2000;96:2310-2313)

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a relentless myeloproliferative disorder of children characterized by the monoclonal overproduction of myeloid cells.^{1,2} Up to 14% of cases occur in children with neurofibromatosis type 1 (NF1),^{3,4} an autosomal dominant disorder caused by germline inactivation of one allele of the *NF1* gene on chromosome 17. JMML can involve more than the myeloid lineage⁵ because a malignant clonal expansion of erythroid cells has been inferred by cytogenetic,⁶ X chromosome inactivation⁷ and microsatellite polymorphic marker studies,⁸ and a JMML patient has been reported whose disease evolved to pre-B-cell acute lymphoblastic leukemia (ALL).⁹ Here we describe a boy with NF1 who was brought for treatment for JMML and in whom a T-cell lymphoma later developed. Molecular genetic and flow cytometric analyses provided strong evidence that both malignant clones derived from a common precursor with pluripotent potential, suggesting that JMML is a stem cell disorder.

Study design

Case report

A 3½-year-old boy with NF1 inherited through the maternal lineage was brought for treatment for JMML. Physical examination showed numerous café au lait spots and enlarged tonsils but an absence of hepatosplenomegaly. His white blood cell count was 96 700/μL, with 4% circulating myelocytes/metamyelocytes, a hemoglobin level of 10.1 g/dL, and a platelet count of 218 000/μL. The bone marrow showed an overwhelming myeloid predominance with less than 5% blasts and a normal karyotype, 46,XY. His peripheral blood myeloid cells formed colony-forming unit granulocyte-macrophage colonies in methylcellulose cultures without exogenous growth factors.

During the next 4 months leukocytosis persisted and was complicated by a worsening anemia and thrombocytopenia with an enlarging spleen, failure to thrive, and airway obstruction caused by hypertrophied tonsils. There was no response to isotretinoin administered at 100-200 mg/m² per day. Adenotonsillectomy and splenectomy were performed, and histopathologic examination of the adenoids and tonsils revealed a dense infiltration with myeloperoxidase-positive cells. Similarly, the enlarged spleen showed expansion of the red pulp by immature myeloid cells. New, diffuse adenopathy and hepatomegaly developed 6 weeks later. A lymph node biopsy revealed a T-cell expansion consistent with lymphoma. He received combination high-dose chemotherapy, but respiratory distress, anasarca, and renal failure ensued, which led to his death 8 months after the diagnosis of JMML.

Flow cytometry

Monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA), DAKO (Carpinteria, CA), and Pharmingen (San Diego, CA). Flow cytometric analysis of the lymphomatous node and bone marrow aspirate was performed as previously described.¹ Using a FACS Vantage (Becton Dickinson), viable cells from an enlarged lymph node were separated into CD4⁺surface CD3⁻ (CD4⁺sCD3⁻) lymphoma cells, and CD4⁺sCD3⁺ (phenotypically normal T cells) populations and viable bone marrow cells were purified as bright CD45⁺ with bright CD5⁺ (phenotypically normal T lymphocytes), bright CD45 with intermediate side scatter (monocytes), and intermediate CD45 with high side scatter (maturing granulocytes) populations. The cells were lysed immediately with DNA preparation buffer (Gentra Systems, Minneapolis, MN) and were snap-frozen in liquid nitrogen.

DNA extraction and analysis for loss of constitutional heterozygosity

DNA was isolated from unfractionated and sorted blood, bone marrow, spleen, and lymph node populations as described.¹¹ To screen for loss of

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heterozygosity (LOH) at *NFI*, 4 intragenic polymorphisms were assayed: EVI-20,¹² an Alu repeat,¹³ a dinucleotide repeat,¹⁴ and a complex repeat.¹⁵ The extent of the chromosome 17 LOH region was determined by assay of polymorphic loci UT172¹² and by 9 loci defined in the Genome Database (<http://gdbwww.gdb.org/>) by their identification numbers—D17S926 (GDB, 199252), D17S805 (GDB, 188452), D17S1294 (GDB, 686175), D17S1800 (GDB, 607032), D17S250 (GDB, 177030), D17S836 (GDB, 1218969), D17S1806 (GDB, 607848), D17S1830 (GDB, 1218973), and D17S928 (GDB, 1218974). Each locus was assayed using the polymerase chain reaction (PCR) to amplify DNA segments that contained a variable number of short nucleotide repeats. Thermocycle parameters and procedures for genotyping each locus have been described.^{12,15} Radiolabeled PCR products were resolved by electrophoresis. Loss and retention of heterozygosity was determined by comparing the alleles detected in the blood of both parents with the allele(s) detected in the patient's tissues.

Results and discussion

LOH for *NFI* served as a marker of somatic inactivation of the normal allele in various hematopoietic compartments and cells showing LOH at *NFI* are likely to be derived from a common precursor cell. Similarly, X chromosome inactivation has been used to demonstrate the clonality of mononuclear cells in girls with JMML.⁷ To test whether the lymphoid and myeloid cells shared a common LOH of *NFI* in this patient, subpopulations of normal and aberrant cells were identified and purified using flow cytometry and subjected to genetic analysis.

Cells from the enlarged lymph node showed 2 populations by forward and side scatter displays (Figure 1). The population of small cells revealed a mixture of phenotypically normal B, T, and natural killer lymphoid cells. The large lymphoma cells (red), which comprised 40% of viable cells, were consistent with T-cell lymphoma. Using surface (s) staining for CD4 and CD3, the T cells were sorted into an abnormal and a phenotypically normal population defined as CD4⁺sCD3⁻ and CD4⁺sCD3⁺, respectively. Cells with this abnormal phenotype could not be identified in the bone marrow (lower limit of detection less than 0.5%). Bone marrow was also used to purify aberrant myeloblasts expressing CD7 and normal T lymphocytes (CD4⁺sCD3⁺), as well as monocytoid forms and maturing neutrophils.

Loss of the normal *NFI* allele inherited from his father was

detected in the patient's blood and bone marrow specimens obtained at the initial diagnosis of JMML (Figure 2A, lanes 3 and 4, respectively). A similar loss of the normal paternal *NFI* allele was identified in the lymph node and in bone marrow cells obtained with the onset of diffuse adenopathy (lanes 5 and 7, respectively), in maturing monocyte and neutrophil fractions purified from the bone marrow (lanes 8 and 9, respectively), and in the immunophenotypically aberrant CD4⁺sCD3⁻ subpopulation of cells purified from the lymphomatous lymph node (lane 11). Unfractionated spleen cells (lane 6) showed a marked reduction in the signal derived from the paternal allele, a result that is consistent with an admixture of *NFI*^{-/-} and *NFI*^{+/-} cells. In contrast, the intensities of the mutant maternal and normal paternal alleles were similar in the phenotypically normal CD4⁺sCD3⁺ T cells isolated from the lymph node (lane 10) and from the phenotypically normal CD5⁺ T cells purified from bone marrow (data not shown), suggesting that these cells were not involved in the malignant process.

To further investigate whether JMML and lymphoma cells derived from a common progenitor, loci spanning the length of chromosome 17 were assayed for LOH. Although multiple loci showed LOH in JMML cells and lymphomatous lymph node cells (CD4⁺sCD3⁻), representative data for the D17S805 locus, which retained heterozygosity, and D17S1294, which lost heterozygosity, are shown in Figure 2B. The loci that lost heterozygosity were identical and spanned the long arm of the chromosome, a large region greater than 50 Mb in length (Figure 2B). In contrast, the normal CD4⁺sCD3⁺ T cells from the lymph node retained heterozygosity at all chromosome 17 loci tested. These data strongly implicate a single genetic event that resulted in loss of the normal paternal *NFI* allele in a progenitor cell that gave rise to both the myeloid leukemia and the lymphoma clones.

The most likely genetic mechanism of LOH in this case is a recombination between D17S805 and D17S1294 of a maternal and a paternal chromatid during the S/G2 phase of the cell cycle of an ancestral cell. All possible recombinants would have 2 apparently normal chromosome 17 homologs, which is consistent with the results of the 2 independent cytogenetic normal analyses of bone marrow from our patient. One recombinant would carry the unaltered *NFI* maternal chromosome and a paternal chromosome in which the 17q arm with the *NFI*⁺ allele had been replaced with

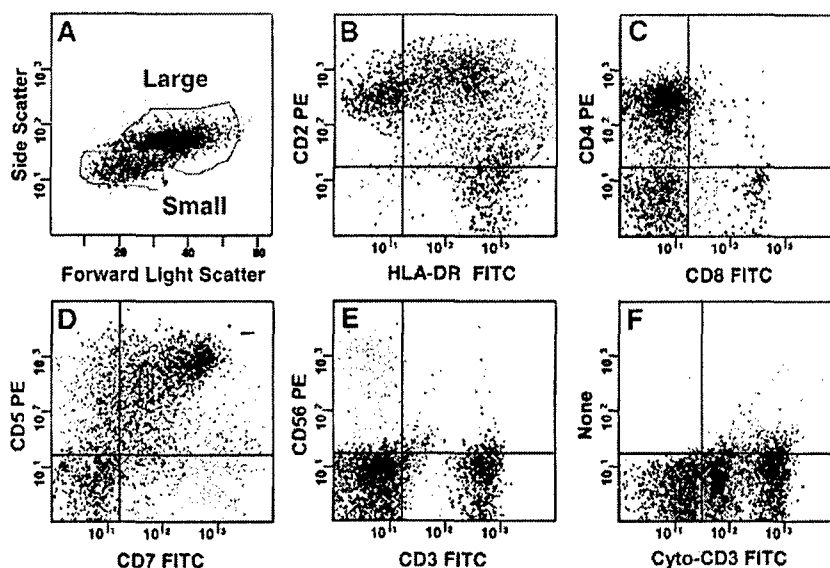


Figure 1. Multidimensional flow cytometric analysis of lymph node cells. Two populations could be defined based on forward and side light scatter (A). The larger T-cell lymphoma population (red) expressed CD2 and HLA-DR (B), CD4 without CD8 (C), and CD5 and CD7 (D). It did not express CD3 or CD56 on the surface (E), but it had CD3 in the cytoplasm (F) and were CD5^{dim}, CD7^{dim}, CD10^{dim}, CD1a⁻, TdT⁻, CD13⁻, and CD34⁻ and did not express B lymphoid markers (data not shown). Small cells in the lymph node contained normal B (green, 20%) and T (blue, 24%) lymphocytes and natural killer cells (gray, 7%).

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Recombination hotspot in *NF1* microdeletion patients

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Neurofibromatosis type 1 (NF1) patients that are heterozygous for an *NF1* microdeletion are remarkable for an early age at onset and an excessive burden of dermal neurofibromas. Microdeletions are predominantly maternal in origin and arise by unequal crossover between misaligned NF1REP paralogous sequence blocks which flank the *NF1* gene. We mapped and sequenced the breakpoints in several patients and designed primers within each paralog to specifically amplify a 3.4 kb deletion junction fragment. This assay amplified a deletion junction fragment from 25 of the 54 unrelated *NF1* microdeletion patients screened. Sequence analysis demonstrated that each of the 25 recombination events occurred in a discrete 2 kb recombination hotspot within each of the flanking NF1REPs. Two recombination events were accompanied by apparent gene conversion. A search for recombination-prone motifs revealed a χ -like sequence; however, it is unknown whether this element stimulates recombination to occur at the hotspot. The deletion-junction assay will facilitate the prospective identification of patients with *NF1* microdeletion at this hotspot for genotype–phenotype correlation studies and diagnostic evaluation.

INTRODUCTION

Haploinsufficiency for neurofibromin, the protein product of the *NF1* gene, causes the autosomal dominant disorder neurofibromatosis type 1 (NF1) (reviewed in refs 1–3). While the majority of cases are caused by subtle private mutations which predict truncation of neurofibromin (4,5), an estimated 5–22%

are heterozygous for a germline deletion spanning the 350 kb *NF1* locus (6–9). Early reports that deletion patients were remarkable for facial anomalies and an early age at onset of cutaneous neurofibromas, or for excessive numbers relative to age in cases for which age at onset was unknown (10,11), have been confirmed by the identification of additional patients (7,9,12–16). A few deletion cases without this phenotype have been reported (7,9,16), but because the extent of the deletions was not delineated, it is unclear whether they involved the same loci. These observations led to the hypothesis that the *NF1* microdeletion resulted in haploinsufficiency for neurofibromin and for the product of a second contiguous locus, which together potentiated neurofibromagenesis (11,17).

This hypothesis was supported by recent data showing that 80% ($n = 17$) of microdeletion breakpoints were clustered at paralogous sequences which flank the *NF1* gene (17). These paralogs, termed NF1REP-P and -M for proximal and medial, respectively, are ~85 kb in length and in direct orientation. NF1REP-mediated deletion most likely occurs by either interchromosomal recombination between misaligned NF1REP elements or intrachromosomal looping-out (17). The analysis of flanking polymorphic loci in family members of affected individuals with *de novo* microdeletions revealed that unequal crossing over during maternal meiosis I occurred in five out of six cases (18). This is consistent with earlier findings that ~80% of *NF1* microdeletions are maternal in origin (6,19).

Other than one expressed pseudogene and four expressed sequence tags (ESTs) (17), nothing is known of putative genes or sequence motifs in the NF1REP elements. Towards elucidating the molecular basis of *NF1* microdeletion and the genes involved, we mapped breakpoints, developed a deletion junction PCR assay, and analyzed the sequences of junction fragments. These analyses identified a hotspot for recombination between the NF1REP-P and -M paralogs.

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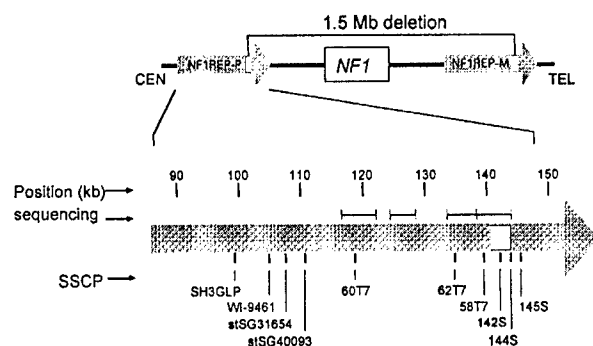


Figure 1. Localization of the *NF1* microdeletion breakpoints. The schematic shows the *NF1* gene and flanking paralogous elements NF1REP-P and -M in direct orientation. NF1REPs and the deleted region are not drawn to scale. *NF1* microdeletion occurs by homologous recombination between these NF1REP elements. The recombination hotspot within each NF1REP is depicted by white boxes. Below, the large gray arrow depicts the generic structure of both NF1REP elements with the position (kb) within the NF1REP shown relative to the sequence of BAC 271K11 (AC005562). PCR-amplified segments that were sequenced are shown above and the 10 loci analyzed by SSCP below.

RESULTS

Refinement of *NF1* deletion breakpoint intervals

Our previous analyses of somatic cell hybrid lines carrying the deleted chromosome of 15 patients with 1.5 Mb deletions showed that in each case the *SH3GLP2* locus in NF1REP-P was retained, while *SH3GLP1* in NF1REP-M was lost (17, and patient C12 in this paper) (Fig. 1). Further refinement of the homologous recombination sites required identifying NF1REP-specific nucleotides. The strategy was to use the known NF1REP-P sequence to design primers to amplify sequences from a somatic cell hybrid line carrying a patient's deleted chromosome 17. Products were analyzed either by direct sequencing or single-stranded conformation polymorphism (SSCP) banding patterns. Results were compared with the sequence or banding pattern of NF1REP-P [AC005562, bacterial artificial chromosome (BAC) 271K11] and the draft sequence of NF1REP-M (AC023278, BAC 640N20; AC021852, BAC 474K4). As summarized in Figure 1, these results identified a common breakpoint interval of ~3 kb in seven of the 15 cases in which somatic cell hybrids were analyzed. As predicted, Southern blot analysis of *BclI*-digested DNA probed with a 200 bp fragment identified a novel deletion junction fragment of ~11 kb in a patient, but not in the patient's healthy parents (Figure 2A). This novel fragment was also detected in three additional unrelated *de novo* microdeletion patients, but not in their healthy parents (data not shown). Some of the breakpoints of the remaining eight hybrid lines carrying deleted chromosomes appear to cluster at a distinct site, but further sequence analysis is required for precise localization.

Detection of a deletion-specific junction fragment by PCR

To assay this recombination site in other *NF1* microdeletion patients, a deletion junction-specific PCR assay was developed. A

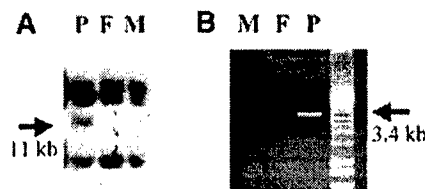


Figure 2. Detection of the *NF1* deletion junction fragment. (A) Southern blot of *BclI*-digested DNA from microdeletion patient 98-1 and his/her healthy parents was probed with a 200 bp PCR product from the breakpoint region of NF1REP-P (AC005562, nucleotides 143567–143766). The arrow indicates the novel 11 kb junction fragment present only in the patient. (B) The deletion junction PCR assay detects a novel 3.4 kb fragment in patient 98-1 but not in his/her healthy parents. F, father; M, mother; P, patient.

forward primer specific for NF1REP-P and a reverse primer specific for NF1REP-M amplified a 3.4 kb junction fragment from DNA of patient C12 and from a somatic cell hybrid line carrying the deleted chromosome of this patient. Specificity of the primers was tested using P1-derived artificial chromosomes (PACs) from the different REPs (NF1REP-M, NF1REP-P, NF1REP-D, PAC with paralogous sequence from chromosome 19p), somatic cell hybrids with a deleted chromosome 17 and control DNA. This assay was then performed on DNA from 54 patients known to carry microdeletions extending beyond the borders of the *NF1* gene. The 3.4 kb deletion junction fragment was detected in 25 of 54 patients, but not in DNA from 75 control subjects. Figure 2B shows an example of this deletion junction PCR in patient 98-1 and his healthy parents. These results document the specificity of the assay in detecting only chimeric NF1REP sequences that arose from this specific deletion event. A chimeric NF1REP consisting of NF1REP-M and NF1REP-D could be excluded because this would not result in an *NF1* phenotype, but in the deletion of about one-third of the long arm of chromosome 17. This was excluded by molecular analysis of the seven somatic cell hybrids and by cytogenetic analysis [including fluorescence *in situ* hybridization (FISH)] in the remaining cases.

Sequence analysis of deletion junction fragments

The sequence of the amplified 3.4 kb deletion junction fragment from 25 positive *NF1* microdeletion patients was determined by direct cycle sequencing. On the basis of available sequences of NF1REP-P, NF1REP-M and our own sequences of this region, we identified 10 REP-specific nucleotide differences (Fig. 3). Analysis of these nucleotides in the patients revealed that the deletion breakpoints were clustered in a 2 kb region of the junction fragment. Fourteen recombination events occurred in the 670 bp segment, two occurred in a 354 bp segment, and seven in a 967 bp segment (Fig. 3). The parental origin of *de novo* deletions was predominantly maternal, but paternal deletions also occurred at this hotspot (patients 99-2 and 940174). Apparent gene conversion events were detected in two unrelated patients (Fig. 3B). The deletions of *de novo* NF1 patients 984412 and 973287 occurred on their maternally-derived chromosomes. The sequence of their mother's NF1REP-P and -M elements identified polymorphisms that facilitated detection of apparent gene conversion events in the patient's chimeric REP which maximally spanned 907 and 317 bp.

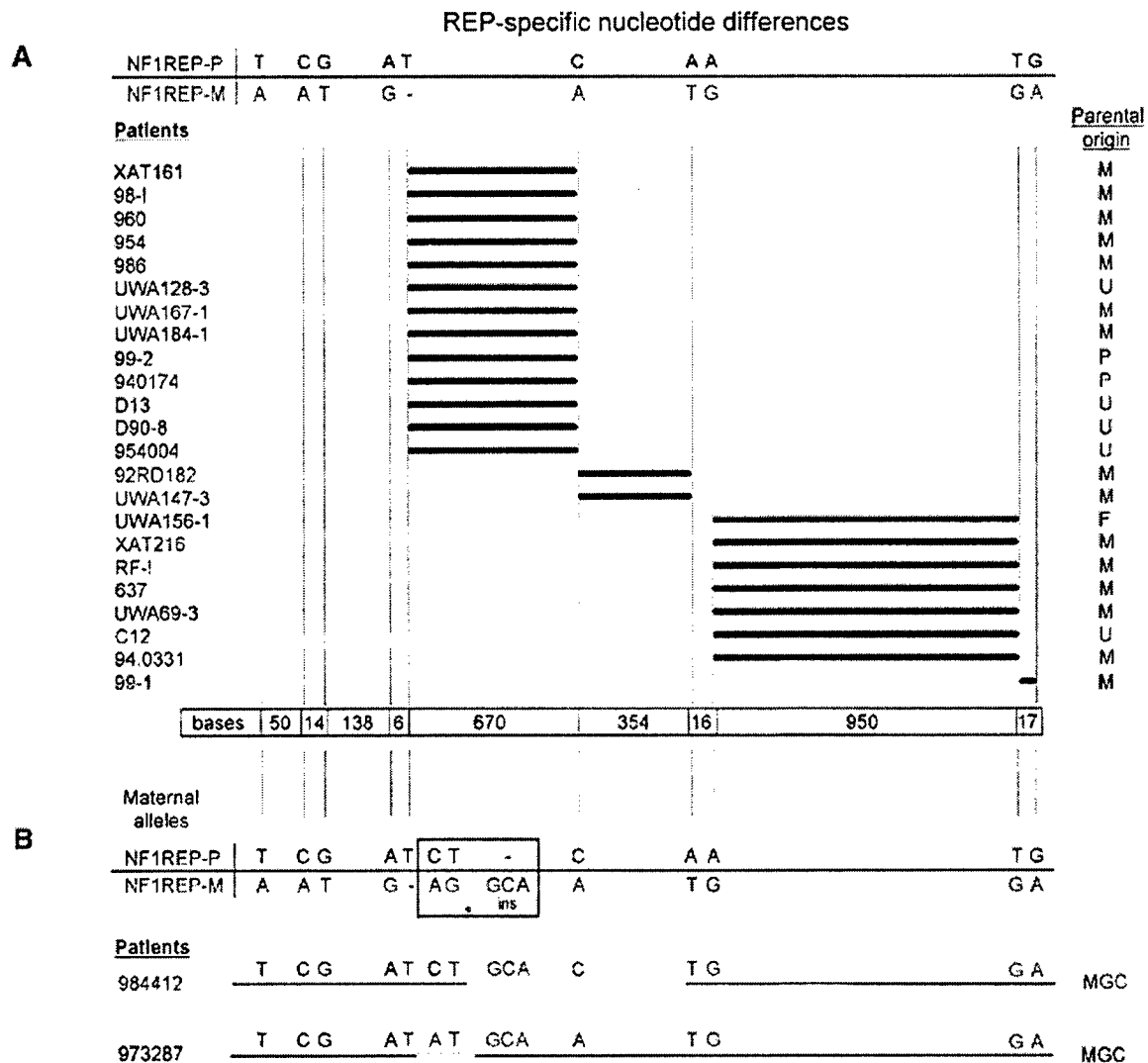


Figure 3. The sites of recombination in 25 *NF1* microdeletion patients. (A) REP-P (black) and -M (red) specific nucleotides in the hotspot region are shown at the top. The position of these nucleotides are indicated below based on the sequence of BAC 271K11 (AC005562) from bases 141624 to 144319. For each patient, the black bar delineates the interval in which the recombination event occurred. The parental origin of the *NF1* microdeletion is indicated at the right: M, maternal; P, paternal; F, familial; and U, unknown. (B) The deletions in patients 984412 and 973287 were accompanied by apparent maternal gene conversion (MGC). The conversions were detected by comparing the sequence of the patient's deletion junction fragment with that of the corresponding regions of the mother's NF1REP-P (black) and -M (red). Both mothers were heterozygous for additional NF1REP polymorphisms (blue box) which facilitated detection of the conversion events. The gene conversion occurred between positions 142110 and 143017 in patient 984412 and between positions 141993 and 142310 in patient 973287 (numbering based on AC005562).

Sequence analysis and structure of NF1REP-P and -M

To investigate the molecular basis of NF1REP-mediated microdeletion, we examined the nucleotide sequence of the REPs for overall sequence identity, GC content, recombination prone motif, and the existence of potentially disrupted transcripts. The structures of NF1REP-P and -M are such that ~60 kb of the >85 kb paralogs are identical in their arrangement (M. Dorschner and K. Stephens, unpublished data). These 60 kb segments are ~98% identical at the nucleotide level, based on comparisons of the finished sequence of

NF1REP-P with the available sequence fragments of the draft sequence of NF1REP-M. Because the sequence quality of NF1REP-M is unknown at this time, the precise degree of nucleotide identity of NF1REP-P and -M may be slightly higher or lower. The nucleotide identity over the entire length of the paralogs appears to be consistent as far as the sequence is available. Pairwise sequence comparisons across the lengths of NF1REP-P and -M did not identify regions with higher or lower nucleotide identities. The average GC content of the NF1REPs is ~50%, while the 2 kb hotspot is ~12% higher.

There is a small region 40–45 kb centromeric to the recombination hotspot with an above average GC content. We have not identified any recombinations in or adjacent to this second region of above average GC content.

A search for recombinogenic motifs and replication-associated sequences in or near the recombination hotspot (Materials and Methods) revealed a χ -like element in the 670 bp recombination interval (Fig. 3; position 142090 of AC005562). WI-12393 is an EST located within each REP (17). A preliminary examination of the gene that includes this EST suggests that it may be disrupted by the deletions described here. It is unknown, however, whether this transcript represents a functional gene or an expressed pseudogene (data not shown).

DISCUSSION

Using an *NF1* microdeletion junction-specific PCR assay, we demonstrated that the recombination event between the NF1REP-P and -M paralogs occurred in a 3.4 kb fragment in 46% of cases with deletions that spanned the *NF1* gene. Sequence and SSCP analyses of the NF1REPs and junction fragments identified REP-specific polymorphisms that enabled us to narrow the breakpoints to a 1889 bp interval near the telomeric end of the NF1REPs (Fig. 1). Within this segment, recombination events clustered in three intervals (Fig. 3). There was no simple correlation between the recombination site and the parental chromosome which underwent deletion. The majority of *de novo* microdeletions occurred preferentially on the maternally-derived homolog, consistent with previous data (6,19). Importantly, however, deletion of paternally-derived chromosomes can also occur in this hotspot region (Fig. 3).

Although a number of deletion/duplication disorders are caused by recombination between flanking paralogs (reviewed in ref. 20), this is only the second to be analyzed at the nucleotide level. The breakpoints have been sequenced for duplications and deletions that cause CMT1A and HNPP diseases, respectively. These two different neuropathies are caused by recombination between flanking CMT1A-REP paralogs. The disease phenotype of CMT1A or HNPP depends upon whether the patient carries a duplication or a deletion of the dosage-sensitive *PMP22* gene located between the CMT1A-REPs (reviewed in refs 21 and 22). There are striking parallels and differences between *NF1* microdeletion and CMT1A/HNPP rearrangements. Each REP-mediated recombination event results in a 1.5 Mb rearrangement, yet the NF1REP is over twice the length of the CMT1A-REP. Both rearrangements show parent-of-origin effects. Eighty percent of *NF1* deletions are maternal in origin and are generated primarily by unequal meiotic crossing over between chromosome 17 homologs (6,18,19). CMT1A duplications are paternal in origin (92%) and also arise by unequal meiotic crossing over between chromosome 17 homologs (23). Maternal rearrangements, albeit CMT1A duplication or HNPP deletion, occur by unequal intrachromatid exchange or excision of an intrachromatid loop, respectively. Both the 85 kb NF1REPs and the 24 kb CMT1A-REPs have discrete recombination hotspots of 2 kb and 557 bp (24), respectively. χ -like sequences are located in or near both recombination hotspots (Fig. 3) (25). In *Escherichia coli*, χ elements stimulate recombination in their general vicinity but whether they can function in a similar manner in humans remains to be verified by experimental data.

In addition, a *mariner*-like transposable element lies ~700 bp from the CMT1A hotspot. This element does not express functional transposase, but it may be a target for a transposase expressed from other such elements in the genome (25). Although recombination hotspots have been identified within each REP element, this does not necessarily imply that these are high frequency meiotic recombination sites in the genome. Recently, a sperm analysis showed that unequal recombination between the CMT1A-REPs occurs at an average rate for the male genome (~1 cM/Mb) (26). It is possible that the special feature of the recombination hotspot region is the combination of a region of high sequence identity and high GC content. However, until now we were unable to find a recombination in the only other region with an above average GC content. Due to the limited availability of sequence from the medial REP (draft quality, unassembled clones) it is impossible at this time to know if other regions of exact sequence identity exist.

Our findings of a recombination hotspot for *NF1* microdeletions and the development of a deletion junction-specific PCR assay have significant implications for research and patient care. *NF1* mutations are typically private and scattered throughout the 8.5 kb coding region, making detection difficult (4). Prior to the findings described here, the most prevalent mutation was R1947X, which occurred in ~1.5% ($n = 255$) of patients and is not associated with any particular phenotype (27). The microdeletion hotspot described here probably accounts for ~5% of *NF1* mutations, based on an estimated microdeletion frequency of 10%. The junction-specific PCR assay will facilitate the identification of the first cohort of NF1 patients with the same mutation. Prospective studies are important to determine whether the deletion is predictive of certain clinical manifestations, such as early age at onset of cutaneous neurofibromas. The majority of *NF1* microdeletion patients in the current study were selected by phenotype. To date, available medical records have confirmed that 10 of the 25 patients with deletions at the hotspot showed an early age at onset of cutaneous neurofibromas (<10 years) or an excessive number of tumors relative to their age. A study to assess the phenotype of the remaining patients is in progress.

Although we anticipate that the deletion junction fragment PCR assay may be clinically useful in some cases of NF1, we consider its implementation at this time to be premature. To date, we have screened 75 healthy individuals with the assay conditions as described. We do not know the frequency of false positives nor how it might be affected by minor alterations in assay conditions. It is possible that this recombination is a low frequency event during mitosis of hematopoietic cells in healthy individuals, which could be detected by our robust and sensitive PCR assay. The probability of detecting such false positives may be higher if the deleted cells have a growth advantage. In addition, this assay cannot differentiate a germline *NF1* microdeletion patient from one with a somatic mosaic microdeletion. There are documented cases of somatic mosaicism for an *NF1* microdeletion, although it is not known whether the recombination events occurred at this hotspot (16,28–30). *A priori*, the germline patient might be expected to have an early onset and a heavy burden of cutaneous neurofibromas, while the somatic might be expected to have a later onset with fewer neurofibromas or other manifestations. In addition, the risk of a mosaic patient having an affected child may be considerably less than that of a germline *NF1* deletion.

patient. Application of the PCR junction fragment assay to the healthy parents of eight of the 17 *de novo* microdeletion patients described here was negative. Although none of our *de novo* deletion patients appear to have a mosaic parent, one such case has been described (16).

It is unclear at this time where the breakpoints of the remaining 54% of *NF1* microdeletions occur. Preliminary data suggests that there may be additional recombination hotspots in the NFIREP elements. The development of junction-specific PCR assays for other putative recombination sites will be important for diagnosis, genotype/phenotype analyses, and understanding the molecular basis for recombination-prone sites in the genome.

MATERIALS AND METHODS

Subjects and cell lines

Peripheral blood samples were obtained after informed consent from 54 *NF1* microdeletion patients and their parents, when available. Previous reports document molecular confirmation of deletion in most of the patients (6,9,12,17). In newly ascertained patients, *NF1* microdeletions were confirmed by both analysis of polymorphic markers and FISH, as described previously (12). In all cases the microdeletion was known to extend beyond the borders of the *NF1* gene. In addition, rodent/human somatic hybrid cell lines carrying only the deleted chromosome 17 homolog were constructed from a subset of patients (11,17).

Fine mapping of *NF1* deletion breakpoints

Breakpoints were mapped in somatic cell hybrids by direct sequencing and/or SSCP of amplified products. For SSCP, 15 µl of loading buffer (0.5% dextran blue, 95% formamide) was added to 15 µl of amplified product, heated at 95°C for 3 min and snap cooled on ice for 1 min. Thirty microlitres was electrophoresed through a 0.5× MDE-gel (FMC BioProducts, Rockland, ME) for 10 h at 4°C, 400 V and visualized by fluorimager after Sybr Green staining. Forward (F) and reverse (R) primers for SSCP analysis were: stSG31654 F, 5'-TGTGAGG-GGCTCTTCTATTG-3' and stSG31654 R, 5'-AGAGTGAT-GTTAGCAGTCA-3'; stSG40093 F, 5'-TGAAGATGTGGA-CCTGCTGA-3' and stSG40093 R, 5'-TGTTGCCAGGCTA-GTTTTT-3'; 60T7 F, 5'-ATCCTCCGCTTTTCTCCT-3' and 60T7 R, 5'-GTTTTAGGGGAGGCCTGTTC-3' (201 bp); 62T7 F, 5'-TGAGAGGCGGGGTGTATTAG-3' and 62T7 R, 5'-TCC-TTCTCCAGCCATTGTTT-3' (187 bp); 58T7 F, 5'-GTATG-GGGAGCTGCTTTTCC-3' and 58T7 R, 5'-TTCTGTGAGA-CCTGGGAAGG-3' (217 bp); 5562-142s F, 5'-TACTCACC-CCTAGGCCACAG-3' and 5562-142s R, 5'-ACACACTCA-GGGACCAACCT-3' (200 bp); 5562-144s F, 5'-TGGCTCCCT-ACTGTGTTTCC-3' and 5562-144s R, 5'-TCACACAGC-GACTCCTTCAC-3' (186 bp); 5562-145s F, 5'-AAATCCCG-GCTTCACAGTTA-3' and 5562-145s R, 5'-GGCTGGTCTCAA-ACTCTTGG-3' (197bp); and WI-9461 (<http://gdbwww.gdb.org/>).

A Southern blot of 10 µg of *BclI*-digested DNA, electrophoresed through 0.6% agarose and transferred to Hybond N⁺ membrane (Amersham, Buckinghamshire, UK), was probed with a 200 bp PCR product from the breakpoint region (5562-142S). The membrane was washed with 2× SSC and

0.1% SDS at 60°C for 30 min, 0.1× SSC and 0.1% SDS at 70°C for 2× 30 min, and exposed to Hyperfilm MP (Amersham) at -70°C for 72 h.

NF1 deletion junction fragment analysis

The 3.4 kb deletion junction fragment was amplified with primers DCF 5'-TCAACCTCCCAGGCTCCCGAA-3' and DTR 5'-AGCCCCGAGGGAATGAAAAGC-3'. A 25 µl PCR was performed using the Expand Long Template PCR System (Roche Molecular Systems, Indianapolis, IN) with 300 ng DNA, 15 pmoles each primer, 0.35 mM dNTPs, 10× PCR buffer 1, and 2.5 U DNA polymerase. After heating to 94°C for 3 min, samples were subjected to 35 cycles of 94°C for 30 s and 68°C for 2.5 min, with a final extension of 7 min at 68°C. Five microlitres of product was electrophoresed through a 1% agarose gel and visualized by EtBr staining.

Junction fragment products were sequenced by cycle sequencing using either the SequiTherm EXCEL II Long Read (Epicentre, Madison, WI) or the Big-Dye Terminator (Applied Biosystems, Foster City, CA) kits. Extension products were analyzed on either an A.L.F. (Automated Laser Fluorescence sequencer, Pharmacia, Uppsala, Sweden) or an ABI 377 sequencer (Applied Biosystems). Raw nucleotide sequences were analyzed with Sequencher (GeneCodes, Ann Arbor, MI), Clustal W (31), and the vector NTI program (Informax, North Bethesda). The nucleotide sequence of the hotspot region was analyzed for a number of recombination prone motifs including: χ from *E.coli* (5'-GCTGGTGG-3'), yeast Ade6-M26 heptamer (5'-ATGACGT-3'), XY32 homopurine-homopyrimidine (5'-AAGGGAGAARG-GGTATAGGGRAAGAGGGAA-3'), retroposon LTR (5'-TCA-TACACCACGCAGGGGTAGAGG-3'), LTR-IS (5'-TGGAAT-CCCC-3'), human minisatellite core sequence (5'-GGGCAG-GAG-3'), two hypervariable minisatellites (5'-GGAGGTGGGC-AGGARG-3') and (5'-AGAGGTGGGCAGGTGG-3'), translin consensus 1 (5'-GCNC[A/T][G/C][G/C][A/T] N₍₀₋₂₎ GCCC[A/T][G/C][G/C][A/T]-3') and consensus 2 (5'-[C/A]TGCAG N₍₀₋₄₎ GCCC[A/T][G/C][G/C][A/T]-3'), and the binding site for the protein *pur* (5'-GGNNGAGGGAGARRRR-3'). In addition, we searched for sequences associated with DNA replication including *Saccharomyces cerevisiae* autonomously replicating sequence (ARS) (5'-WTTTATRTTTW-3'), *Schizosaccharomyces pombe* ARS consensus (5'-WRTTTATTTAW-3'), consensus scaffold attachment regions (5'-AATAAAYAAA-3', 5'-TTWTWTTWTT-3', 5'-WADAWAYAWW-3' and 5'-TWWT-DTTWWW-3'), topoisomerase II binding site (5'-GTNWAYAT-TNATNNR-3'), and human replication origin consensus (5'-WAW-TTDDWWWDHWGWHMAWTT-3'). The recombinogenic and DNA replication-associated motifs were described previously (32 and references therein).

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LETTER TO JMG

Independent *NF1* mutations in two large families with spinal neurofibromatosis

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The neurofibromatoses are a group of neurocutaneous disorders that show extreme clinical heterogeneity and are characterised by growth abnormalities in tissues derived from the embryonic neural crest.^{1,2} Two main clinical forms exist, type 1 (NF1) and type 2 (NF2), as well as several alternate and related forms.^{2,3} NF1 and NF2 are the only clinically well defined disorders and both genes have been identified.⁴⁻⁶ The NIH diagnostic criteria for NF1, as defined by the conference statement,⁶ are met if two or more of the following are found: six or more CAL spots; two or more neurofibromas of any type or one plexiform neurofibroma; axillary or inguinal freckling; optic glioma; two or more Lisch nodules; a distinct osseous lesion; a first degree relative (parent, sib, or offspring) with NF1 according to the above criteria.

Spinal nerve sheath tumours are described as symptomatic findings in only 5% of NF1 patients,¹⁰ although they can be observed by MRI in up to 36% of patients.¹¹⁻¹³ The presence of a wide, symmetrical distribution of spinal neurofibromas, occurring in all adult affected members of the same family and segregating in an autosomal dominant fashion, is however extremely rare. This form, familial spinal NF (FSNF), has been considered an alternate form of NF since patients generally lack dermal neurofibromas and Lisch nodules, both typical hallmarks of NF1, and since symptomatic and generalised spinal neurofibromas are uncommon in classical NF1. FSNF has been reported in only four families.¹²⁻¹⁵ Three multigenerational families with spinal neurofibromas and CAL spots were shown to be linked to markers surrounding the *NF1* locus.¹²⁻¹⁵ In the fourth family, presenting with spinal neurofibromas without CAL spots, linkage to the *NF1* locus was excluded.¹⁴ Only in one FSNF family has the underlying molecular defect been documented so far,¹⁵ which was a unique frameshift mutation 8042insA in *NF1* exon 46. Here we describe the identification of the *NF1* mutation in both remaining FSNF families originally described by Pulst *et al.*¹⁴ and Pöyhönen *et al.*¹² Our current findings emphasise that FSNF (with CAL macules) is caused by mutations in the *NF1* gene, but does not support the hypothesis that it is caused by a unique type of *NF1* mutation.

MATERIALS AND METHODS

Subjects

The pedigrees of the families studied are shown in figs 1A and 2.

Skin samples of controls were obtained from operations carried out for cosmetic reasons from four healthy persons at the Department of Dermatology, University of Oulu, Finland, with the consent of the Ethical Committee of Oulu University Hospital.

NF1 mutation analysis

Epstein-Barr virus (EBV) lymphoblastoid cell cultures from two affected members of family 1 and fibroblast cell cultures from two affected members of family 2 were treated with and

Key points

- Familial spinal neurofibromatosis (FSNF) is considered to be an alternate form of neurofibromatosis, with a limited phenotype of multiple spinal nerve root neurofibromas and café au lait (CAL) macules in all affected adults. Only three multigeneration families with FSNF have been reported and in one of them the genetic defect was identified previously: 8042insA in exon 46 of the *NF1* gene.
- As NF1 is notorious for its extreme phenotypic variability even within the same family, the striking homogeneity of the phenotype in these families suggests that a particular type of mutation might underlie this condition.
- We identified the *NF1* mutation in the remaining two FSNF families whose phenotype was known to be linked to chromosome 17q11.2 markers. In the first family, a novel substitution at the splice donor site of exon 39 (7126+3A>C), leading to skipping of exon 39, was found. In the second family, a missense mutation L357P in exon 8 was identified as the sole alteration. The mutation segregates with the disease in both families.
- The current findings emphasise that FSNF (with CAL macules) is caused by mutations in the *NF1* gene, but does not support the hypothesis that it is caused by a specific type of *NF1* mutation. It is anticipated that expression studies and mutation analysis, in the first instance of the genes tightly linked to the *NF1* locus, in both families may lead to the detection of (a) modifier(s) for this specific phenotype.

without puromycin before total RNA extraction as described previously.¹⁶ DNA was extracted from EBV cell cultures or fibroblasts from all family members.

The optimised PTT for the entire coding region was applied essentially as described previously with abnormal fragments further analysed by cDNA and genomic sequencing.¹⁶ Then, for both families, the entire *NF1* cDNA was sequenced using dye-primer chemistry on an automatic genetic analyser (ALFexpress). Information on the sequencing primers used is available upon request.

Western blot analysis

Western blot analysis, using an anti-NF1 antibody (NF1GRD(D)) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and peroxidase linked donkey anti-rabbit (NA 934) (Amersham International plc, Little Chalfont, Buckinghamshire, England) as a secondary antibody, was carried out essentially as previously described.¹⁷

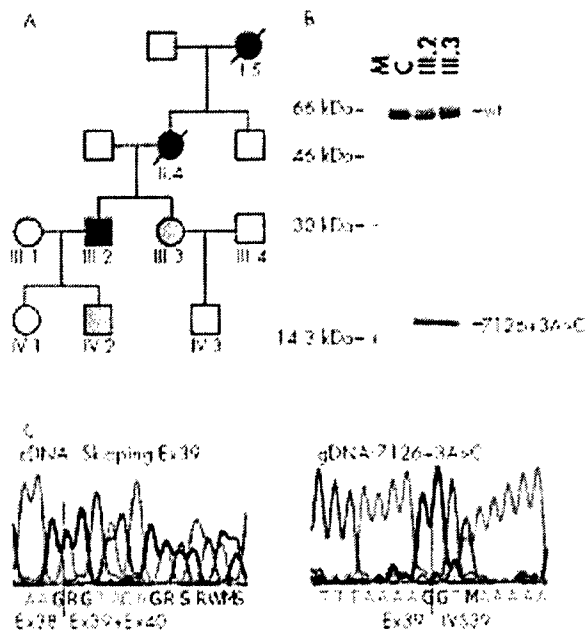


Figure 1 Pedigree and PTT results of family 1 with mutation 7126+3A>C. (A) In the pedigree, blackened symbols denote affected subjects with spinal neurofibromas and CAL spots, grey symbols denote affected subjects with CAL spots only, and white symbols denote healthy subjects. (B) PTT results using primers encompassing exons 34 to 49 of a control (C) and of patients III.2 and III.3. The wild type (wt) and aberrant band caused by skipping of exon 39 are shown. M denotes a protein marker with sizes in kDa. (C) Direct cycle sequencing of mutant cDNA transcripts and genomic DNA. By direct cDNA sequencing of the patient, heterozygosity for transcripts containing exon 39 and transcripts in which exon 39 is skipped are seen. In the genomic DNA, heterozygosity for A and C at position +3 of the splice donor site of exon 39 is seen in the patient.

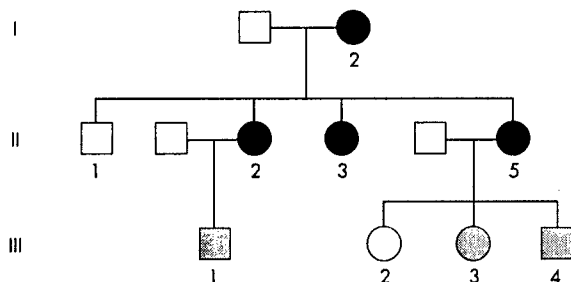


Figure 2 Pedigree of family 2 with mutation L357P. In the pedigree, blackened symbols denote affected subjects with spinal neurofibromas and CAL spots, grey symbols denote affected subjects with CAL spots only, and white symbols denote healthy subjects.

RESULTS

Clinical evaluation

In both families all affected adults, except for one 45 year old female in family 1 (III.3) showed multiple symmetrically distributed spinal neurofibromas in the cervical, thoracic, and/or lumbar region (fig 3). All 12 affected members in both families also had more than six (size over 15 mm) cutaneous CAL spots, but no iris Lisch nodules were present. Clinical data, originally described by Pulst *et al*¹⁴ and Pöyhönen *et al*,¹² have been updated and are summarised in table 1.

Molecular analysis

In family 1, PTT analysis of the *NF1* cDNA of patients III.2 and III.3, encompassing exons 35 to 49, showed in addition to the

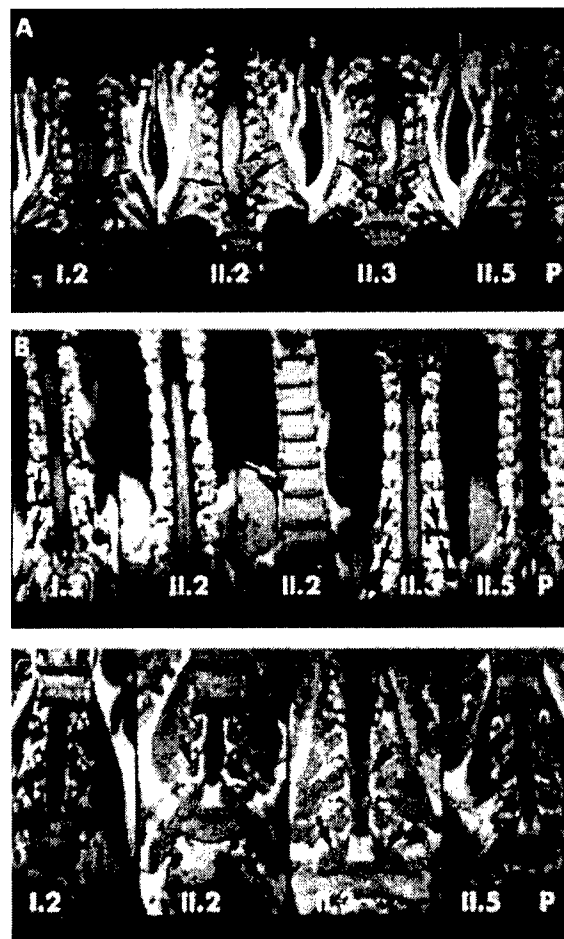


Figure 3 Coronal T1 weighted non-contrast MR images (Siemens Magnetom 1.0T, SE, TR 500, TE 15, slice thickness 3 mm) of patients I.2, II.2, II.3, and II.5 (P-proband) from family 2, carrying the mutation L357P. (A) Cervical spine. (B) Thoracic spine. (C) Lumbar spine. Several extradural, intraspinal neurofibromas compressing the spinal cord (black curved arrows) and extraspinal neurofibromas (black straight arrows) are seen in all patients. Not all tumours are marked. II.2 also has a right sided paraspinal thoracic mass (white arrow). The intra- and extraspinal components of a dumb-bell tumour in the lumbar spine (thick black arrow) in patient II.3 are shown.

wild type 68.5 kDa product a truncated polypeptide of approximately 19 kDa, suggesting a truncating mutation between nucleotides 6900 and 7200 (fig 1B). Sequencing of the RT-PCR product showed absence of the whole of exon 39 (fig 1C). By genomic DNA sequencing, a novel substitution, 7126+3A>C (fig 1C), was found at the splice donor site of exon 39. This is a novel mutation not identified previously in classical *NF1* patients. We calculated the splice site donor strength from the wild type sequence (7126+3A) and the mutant sequence (7126+3C) using the algorithms developed by Shapiro and Senecapathy¹⁸ (S&S) and using the Splice Site Prediction by Neural Network (SSPNN, http://www.fruitfly.org/seq_tools/splice.html) program. The scores for the mutant sequence (69.9 (S&S) and 0.09 (SSPNN)) were significantly lower than the wild type sequence scores (79.9 (S&S) and 0.82 (SSPNN)). Hence, less efficient binding of splice factors at the mutant splice donor site will modulate the splicing efficiency leading to skipping of exon 39. In order to exclude the presence of a putative second alteration in the *NF1* gene, the complete *NF1* cDNA was sequenced using direct cycle sequencing, but no other pathogenic alteration was

Table 1 Clinical features of affected patients in FSNF families 1 and 2

	Family 1					Family 2								Totals
	I.5	II.4	III.2	III.3	IV.2	I.2	II.2	II.3	II.5	III.1	III.3	III.4		
Age (now)	Died at 70	Died at 56	46	45	15	68	45	43	39	9	8	7		
>6 CAL (>15 mm)	+	+	+	+	+	+	+	+	+	+	+	+	12/12	
Spinal neurofibromas	+	+ T	+ L, T	-	?	+ C, T, L	+ C, T, L	+ C, T, L	+ C, T, L	ND	-	-	7/10	
Cutaneous/plexiform neurofibromas	ND	-	-	-	?	-	-	4*	1†	-	-	-	2/10	
Lisch nodules	ND	-	-	-	?	-	-	-	-	-	-	-	0/10	
Freckling	ND	-	-	-	?	+	-	-	-	-	-	-	1/10	
Paraparesis	+	-	+	-	-	+	Weakness	Weakness	Weakness	-	-	-	6/12	
Other findings							MRI‡							

C=cervical; T=thoracic; L=lumbar; ND=no data.

III.3 did not have any spinal neurofibromas 10 years ago, current status unknown.

*One histology proven neurofibroma. †Not biopsied. ‡5 mm tumour right frontal lobe. Unidentified bright object? Cerebral peduncle?

found. 7126+3A>C was present in all affected subjects (II.4, III.2, III.3, and IV.2) and was absent in the healthy family members (III.1, III.4, IV.1, IV.3).

In family 2, only normal sized fragments were discerned by PTT analysis of the total coding region, indicating the absence of a truncating mutation. Later, direct cycle sequencing of the total *NF1* cDNA was performed and a missense mutation was identified in exon 8 as the sole alteration, changing leucine at amino acid 357 to proline (L357P). Patients I.2, II.2, II.3, II.5, III.1, III.3, and III.4 were shown to bear the mutation L357P, whereas the healthy family members II.1 and III.2 did not carry this mutation. This indicates that the mutation segregates with the disease. Furthermore, this alteration was not found by analysing 200 normal chromosomes. L357 is also conserved in *Fugu rubripes* (AF064564), *Drosophila* (L26501), mouse (L10370), and rat (D45201). Proline is a very rigid amino acid and its presence creates a fixed kink in a polypeptide chain, which might have dramatic consequences for the protein structure. Altogether, these data indicate that L357P is the pathogenic *NF1* mutation in this family.

Western blot analysis

Four fibroblast cell cultures from normal subjects and three fibroblast cell cultures initiated independently from the normal skin of patient II.5 carrying the missense mutation L357P (family 2) were analysed by Western transfer analysis. This showed that the levels of NF1 protein were slightly higher in three out of four fibroblast cell lines from normal subjects compared to fibroblasts cultured from patient II.5 (data not shown).

DISCUSSION

Familial spinal NF has been considered an alternate form of NF1 because of the exceptional occurrence of multiple spinal neurofibromas affecting spinal roots symmetrically in all affected adult members of the same family accompanied by very mild cutaneous signs of NF1 and absence of Lisch nodules.¹ Only three such well documented families have been described and CAL spots were considered the sole cutaneous sign present in all affected members.¹²⁻¹⁴ Although the clinical manifestations in the family members fulfil the NIH criteria for NF1 (presence of more than six CAL spots, two neurofibromas of any type, in this case multiple spinal neurofibromas, and also a first degree relative (parent, sib, or offspring) with NF1), they are very atypical of NF1. First, freckles, dermal neurofibromas, and Lisch nodules are found in more than 80%, 90%, and 90% respectively of classical NF1 patients but these signs were generally absent in all affected members of the FSNF families studied. One affected member had freckles in the axillary area and two had dermal neurofibromas, but no Lisch nodules were seen. Secondly, symptomatic spinal neurofibromas are rare in NF1 patients (<5%). Furthermore,

the presence of multiple spinal neurofibromas affecting all spinal roots symmetrically in affected patients leading to severe neurological impairment is generally atypical of NF1, a disease noted for its variable expressivity among family members with identical *NF1* mutations. These very specific clinical findings suggest that a genotype-phenotype correlation might be found in these families and that a specific type of gene defect with a special effect on neural crest cells and/or their precursors in the nerve roots might be present in these families.

In one of these families, the pathogenic lesion had been identified previously, a unique frameshift at the 3' end of the *NF1* gene in exon 46, 8042insA.¹⁵

We have now identified a bona fide pathogenic mutation in the affected members of both the other families. In family 1, a splice donor mutation was identified (7126+3A>C) resulting in out of frame skipping of exon 39 at the mRNA level. This is a novel mutation not identified previously in classical NF1 patients. In family 2, a recurrent missense mutation in exon 8 was found (L357P). This mutation was reported previously¹⁶ in an NF1 patient, but no clinical findings are available so we are unable to correlate this genotype with the specific phenotype.

The current findings underline that FSNF with CAL spots is not only linked to the region flanked by the markers HHH202 and pEW206, encompassing the *NF1* locus, but is clearly allelic with NF1. Furthermore, taking the data from Ars *et al*¹⁵ and ours together, it is clear that all three well defined FSNF families carry a different private *NF1* mutation, as also is the case for classical NF1. Moreover, it is unlikely that a specific type of mutation located in a specific region of the gene underlies this phenotype, as a frameshift, a splice mutation, and a missense mutation were found, all located in different parts of the gene. However, it is noteworthy that the mutations found in the three FSNF families might be mild mutations with some residual function as they are a truncating mutation at the very 3' end of the gene 8042insA, a splicing error 7126+3A>C, and a missense mutation L357P. Recently, Kaufmann *et al*¹⁷ described two families with multiple spinal tumours without CAL spots. Although overall the cutaneous signs were very mild in the affected patients, several of them had cutaneous neurofibromas. In these families too, the underlying *NF1* mutation was a splicing (IVS31-5A>G) and a missense mutation (L2067P). It has been proposed that the FSNF phenotype might arise through a negative residual function of the mutant neurofibromin with a special effect on the development of the neural crest cell.¹⁸ When a total gene deletion or a nonsense mutation in the gene is encountered, these mutations will result in a null allele or in transcripts that become degraded by the nonsense mediated RNA decay, leading to haploinsufficiency. For the mutations found in the FSNF families, however, it is conceivable that a negative residual function might still exist, that is, that a mutant neurofibromin with the single amino acid change is formed by the L357P allele, a 2680 amino acid truncated protein is formed

by mutation 8042insA, and a 2353 amino acid truncated protein is formed by 7126+3A>C, albeit probably in a minimal amount. This assumption was tested by western blotting of the EBV cell lines of two patients carrying 7126+3A>C, using the antibody SC67, but no shortened neurofibromin was found (data not shown). However, we do not think that this is enough evidence to draw definitive conclusions as the molecular tools available for these studies are still limited today.

Mild mutation can however not be the sole factor predisposing to the development of multiple spinal tumours, as a number of similar frameshift mutations at the 3' end of the *NF1* gene, splicing, and missense mutations were found in patients without spinal involvement^{16-19, 21} (unpublished data) and with classical cutaneous manifestations. It is generally accepted that bona fide missense mutations may point to critical functional domains in a protein¹⁹ as they may lead to the production of a mutant protein. The RasGAP activity of the central GAP related domain as well as the structure of the GRD of neurofibromin have been well characterised^{22, 23} and the effects of specific missense mutations in this domain have been studied in detail.²⁴ No functional significance has been contributed so far to the region encompassing exon 8 in which the L357P mutation was found. Recently, the neurofibromin content of cells from three NF1 patients with spinal neurofibromas without CAL spots was found to be reduced to half of the amount of normal control cells, suggesting functional haploinsufficiency.²⁰ Interestingly, one of the mutations tested was a missense mutation L2067P. In order to substantiate these findings further, we have performed western blot analysis on control normal fibroblast cell cultures (n=4) and fibroblast cell cultures (n=3), independently initiated from patient II.5, carrying the mutation L357P. The levels of NF1 protein were slightly higher in three out of four control normal fibroblast cell lines compared to the L357P cell lines. No apparent differences were noted between fibroblasts from one normal control compared to patient II.5 with FSNF. This most probably is because the *NF1* gene is not a housekeeping gene and the same cell line can display marked expression changes when analysed at different times.²⁵ However, we do not think that this evidence is enough to form a definitive conclusion of haploinsufficiency. It was also proposed that the FSNF was the result of the presence of a second *NF1* mutation, either on the normal allele (in *trans*) or, more likely, on the same allele that also contains the already identified mutation (in *cis*).¹⁵ As we applied the complete cascade of techniques used to find the mutation in >95% of classical NF1 patients¹⁶ and did not find a second alteration, it is unlikely that a second change in the *NF1* gene is present concomitantly.

Finally, it is possible that an additional mutation in another gene lying within the region flanked by the markers HHH202 and EW206 and shown to be linked in both families influences the striking clinically homogeneous phenotypic outcome. Analysis of additional highly polymorphic microsatellite markers in both families will allow narrowing down of the linked region of interest for further investigation. It has been suggested that a modifier gene resides in close proximity to the *NF1* gene²⁶ and that its deletion together with the *NF1* gene results in the severe "*NF1* microdeletion" phenotype with early onset of growth and an excessive number of cutaneous neurofibromas. A mutation (for example, with a dominant negative effect on the development of the neural crest cells that are responsible for the NF1/FSNF phenotype) in one of the genes flanking the mutant *NF1* gene might cooperate with the *NF1* mutation to result in FSNF. Furthermore, the same type of alteration, present in *trans* with an *NF1* mutation or occurring as a somatic event may be the modifier causing spinal tumours in a proportion of NF1 patients, but not in their offspring carrying only the *NF1* mutation. Expression and mutation analysis of the genes residing in this region, in the patients from FSNF families and in isolated patients presenting with this specific phenotype, will shed more light on this hypothesis

and should help in understanding the molecular alterations that cause this severe neurological phenotype.

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The development of additional competitive quantitative PCR assays. We developed additional assays spanning the *NF1* gene region. The assay for each locus were screened against an appropriate panel of monochromosomal human-rodent hybrid cell lines to ensure we are not amplifying homologous loci from other chromosomes. Location of the loci are shown in Figure 6

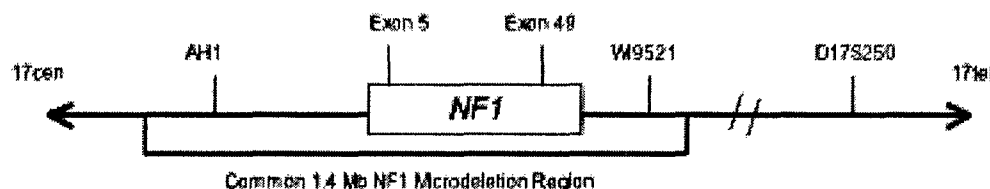


Figure 6. Location of loci in the 17q11.2 *NF1* region for which competitive, quantitative PCR assays were developed. Two loci are intragenic to *NF1* and two are flanking; all four are within the common 1.4 Mb *NF1* microdeletion region previously defined by us (8, 15). D17S250 is at 17q22 region and is never involved in *NF1* microdeletions; it is used as a disomic control locus.

The intra-assay and inter-assay variation of these assays is minimal as shown in Table 1 (Wang B et al., unpublished data). There is no overlap of values for disomic samples vs. monosomic samples. As expected, the disomic control locus D17S250 gave values of approaching 1.0 for both subjects.

Table 1. Variation of competitive quantitative PCR assays¹

Intra-assay variation (mean \pm 1 S.D.)					
Locus	AH1	<i>NF1</i> exon 5	<i>NF1</i> exon 49	WI-9521	D17S250
Normal subject	0.97 \pm 0.18	1.21 \pm 0.13	1.03 \pm 0.1	1.10 \pm 0.15	1.11 \pm 0.16
<i>NF1</i> microdeletion subject	0.33 \pm 0.05	0.52 \pm 0.13	0.51 \pm 0.11	0.44 \pm 0.13	1.15 \pm 0.18
Inter-assay variation (mean \pm 1 S.D.)					
Normal subject	0.95 \pm 0.13	1.23 \pm 0.26	0.97 \pm 0.16	0.98 \pm 0.17	1.07 \pm 0.15
<i>NF1</i> microdeletion subject	0.32 \pm 0.13	0.53 \pm 0.14	0.48 \pm 0.06	0.36 \pm 0.10	1.12 \pm 0.14

¹Intra-assay variation was determined by assay of 20 replicate samples in one experiment. Inter-assay variation was determined by assay of the same normal subject and deletion subject in 20 independent experiments performed on different days.

We have just completed screening several hundred *NF1* subjects for deletions involving the *NF1* locus using these assays (Wang B et al., unpublished data). Some of the deletions are still being confirmed by fluorescence

ratio of peak area of normal control (2 copies) over the peak area of the patient target DNA is calculated, see below. (Wang B et al., unpublished data).

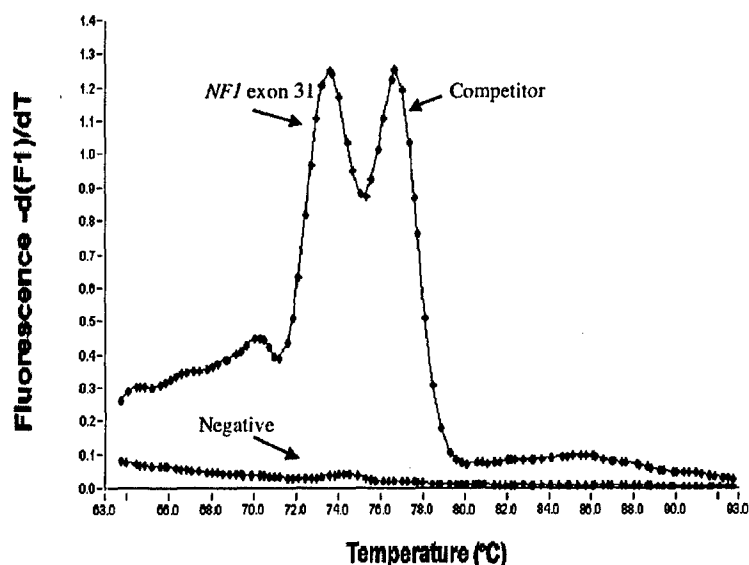


Figure 3. Melting curve analysis after competitive, quantitative PCR at NF1 intron 31 in genomic DNA of a normal control individual. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplification. (Wang B et al., unpublished data).

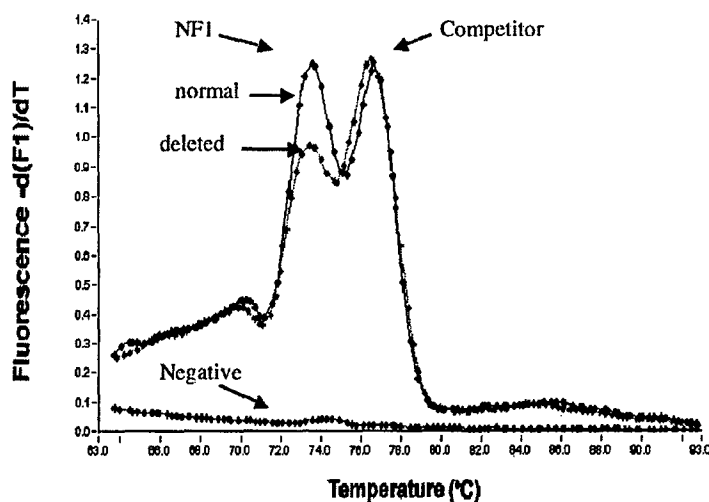


Figure 4. Melting curve analysis after competitive, quantitative PCR at NF1 intron 31 can differentiate one gene copy versus two gene copies. The results of two reactions are shown, closed circles represent target DNA from a normal control individual and closed squares represent target DNA from an NF1 patient with a deletion of one gene. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplification. (Wang B et al., unpublished data).

Once the concentration of competitor is determined for a certain concentration of the normal control DNA, it is essential that all subsequent reactions with unknown patient DNA samples contain exactly the same

concentration of target DNA. Prior to the competitive quantitative PCR assay, we determine the exact concentration of each patient sample using real-time quantitative PCR at a different locus. We amplify the TPA (tissue plasminogen activator) gene on chromosome 12 in each patient and compare that to a standard curve using the normal control DNA. From this reaction, we can calculate exactly what volume of patient DNA must be added to the competitive quantitative PCR assay. An example of the TPA real-time PCR and standard curve is shown in Figure 5. (Wang B et al., unpublished data).

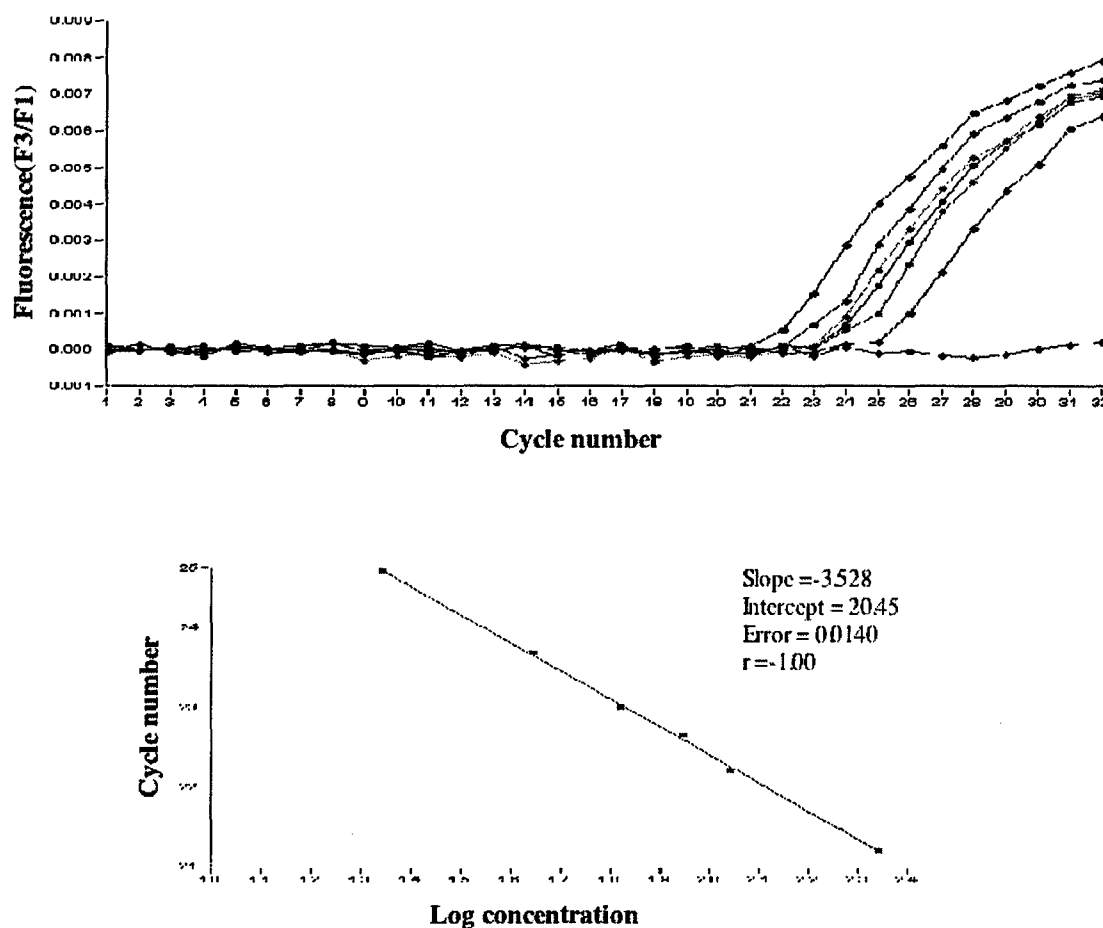


Figure 5. LightCycler real-time PCR at TPA locus showing standard curve. The upper panel shows the results of real-time PCR of the TPA locus of a dilution series of a normal control individual. The reaction consists of unlabeled primers and uses an internal labeled (fluorescence resonance energy transfer (FRET) probe for detection of product. The crossing point (Ct) is defined as the fractional cycle at which fluorescence begins to increase exponentially and is calculated by the LightCycler. Ct becomes larger as the number of TPA targets decreases. The lower panel shows the standard curve calculated from the above data. Note the low error and high correlation coefficient. (Wang B et al., unpublished data).